STUDIES ON α-1,4-GLUCOSANS

- by -

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ABSTRACT OF THESIS

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Title of Thesis Studies on α-1,4- Glucosans

1. Structural studies have been carried out on the branched α-1,4-glucosans, amylopectin and glycogen.

By periodate oxidation, the percentage of 1,6-linkages in these polysaccharides has been measured. The degradation of α-1,4-glucosans by salivary α-amylase has been related to this value, and has been used to measure the average chain length of very small amounts of material.

β-Amylolysis has enabled the average lengths of exterior and interior chains to be calculated; these increased in the order, glycogen β-dextrin, invertebrate glycogen, mammalian liver glycogen, mammalian muscle glycogen, amylopectin. The iodine staining power was found to increase in the same order; the iodine binding of glycogen was found to be different from that of amylopectin, the latter being independent of exterior chain length.

Glycogen reacted with the globulin, concanavalin-A, the intensity of the reaction being indirectly proportional to the exterior chain length. Neither amylopectin nor amylopectin β-limit dextrin gave any reaction; it was concluded that the reaction depended on the interior branching characteristics of the molecule.

2. The molecular structure of a series of horse and ox muscle glycogens isolated during the pre- and post- stages of <u>rigor mortis</u> has been studied by the above-mentioned methods. The exterior chain length of ox sternocephalicus muscle glycogen (post-rigor) was significantly shorter than that isolated from this muscle in the pre-rigor state.

3. Four cases of glycogen-storage disease have been studied. The glycogens from two of the cases had normal structures, but those from the other two cases had unusually short exterior chains. The former cases were due to a low level of activity of glucose-6-phosphatase,

Use other side if necessary.

the latter were due to a decreased activity of the debranching enzyme system.

4. The Z-enzyme activities of barley and 'stock' soya-bean β-amylase, which cause complete β-amylolysis of amylose, have been found to be due to traces of α-amylase. Both α-amylase contaminants are stabilized by calcium ions. Limited action of these enzymes on glycogen β-dextrin has been ascribed to their low concentration, coupled with the reduced affinity of α-amylases for branched substrates.

The selective destruction of Z-enzyme in β-amylase preparations from soya-beans has been partly accomplished by the removal of calcium ions.

5. The action of potato P-enzyme on α-1,4-glucosans has been investigated, the activity towards various substrates decreasing in the order, amylose, amylopectin, glycogen. In the case of branched polysaccharides the limit of P-enzyme action is, in general, less than the muscle phosphorylase limit and the β-amylase limit.

Oxidation of amylose has been found to introduce barriers to P-enzyme action. These barriers may also be resistant to attack by β-amylase.

ACKNOWLEDGEMENTS

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A.W.

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GENERAL INTRODUCTION

Naturally occurring carbohydrates include the polysaccharides which are derived from the condensation of a large number of monosaccharide units. In Nature they provide structural materials which are relatively rigid and almost biochemically inactive. They also act as reserves of monosaccharide units in which case they are continually being metabolized. This thesis is concerned with the structural analysis of three polysaccharides using purified enzymes in conjunction with certain chemical and physico-chemical methods; the three polysaccharides are amylose and amylopectin, the two components of starches, and glycogen, the reserve carbohydrate of animal cells. All three polysaccharides are α-1,4-glucosans, i.e. they consist mainly of α-1,4-linked D-glucopyranose residues, but they differ with regard to degree of polymerization (DP), and the proportion of minor structural linkages; they therefore have different physical properties.

As an introduction to this work, an account of the main structural features of the α-1,4-glucosans, and the enzymes which are involved in their synthesis and degradation will be given.

A. Structure of Starch and Glycogen

1. Starch

Starches are the principal food reserves in many plants; they occur as discrete granules whose characteristics vary from one plant to another. It is generally accepted that most starches contain two main components, amylose and amylopectin, which are structurally different although it has been suggested that starch is a giant homogenous molecule and that the apparent separation into components is due to a hydrolytic or degradative effect (1).

(a) Amylose

Amylose, the minor component of most starches, is an essentially linear molecule with a DP which may vary from ca. 50 to 6,000 according to the plant source and methods of extraction.

There is evidence from periodate oxidation (2), enzymic degradation studies (3,4) and physico-chemical measurements on hydrodynamic behaviour (5) which suggests the possibility of either a low degree of branching or the presence of structural anomalies in the polymer chain, although linkages other than the α-1,4-glucosidic type probably do not represent more than a fraction of one percent of the total. The significance of these observations will be discussed in detail in Section 5.

(b) Amylopectin

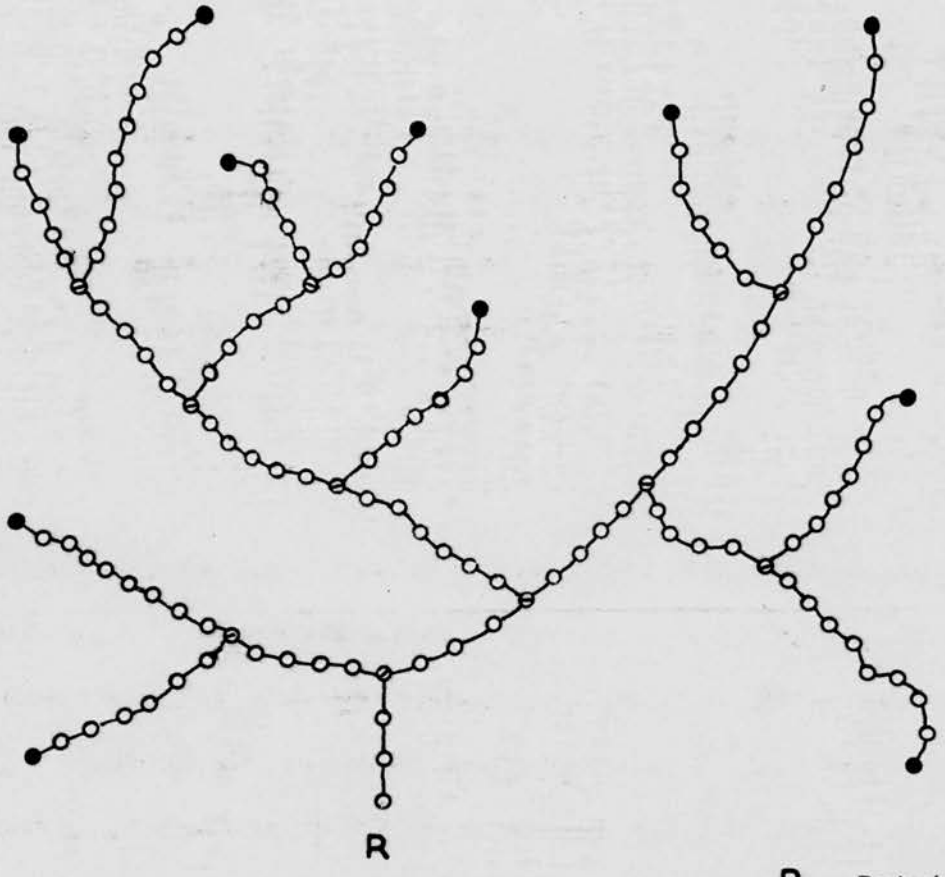
Amylopectin is the major component of most starches and

virtually the sole component of the waxy cereal starches. The molecule is composed of several hundred to several thousand chains of α-D- glucopyranose residues joined through 1,4 -linkages, which are inter-linked in random fashion to form a branched structure. Most amylopectins contain 4-5% of inter-chain linkages, which represents on a statistical basis an average chain length of 25-20 glucose residues.

Methylation (6) and periodate oxidation (7) studies show that the majority of the inter-chain linkages are of the 1,6 - type. An α -configuration for this linkage is indicated by the isolation of isomaltose (0 - α - D - glucopyranosyl-(1$\rightarrow$6)- 0 - D-glucopyranose) and panose (0 - α - D -glucopyranosyl - (1 $\rightarrow$6) -0 - α -D - glucopyranosyl-(1 $\rightarrow$4)-0 - D - glucopyranose) from partial acid hydrolysates of amylopectin (8,9,10), and more recently, by the separation of 6^3- α -maltosylmaltotriose from the products obtained by the salivary α - amylolysis of waxy maize starch (11).

The presence of other types of linkages in amylopectin has been suggested. Nigerose (0 - α - D - glucopyranosyl-(1$\rightarrow$ 3) - 0- D- glucopyranose) has been isolated in very small yield from partial acid hydrolysates (12). The presence of glucose (ca. 0.2%) in the hydrolysates of the polyalcohol from periodate-oxidized amylopectin may also indicate the presence of 1$\rightarrow$ 3 or possibly 1$\rightarrow$ 2 linkages (13). These facts, together, seem to prove the presence of small numbers of α -1,3 -glucosidic linkages; however in the first case nigerose

FIGURE 1.



R Reducing end-group

● Non-reducing end-group

○ Glucose residue linked in the 1 and 4 positions

⊘ Glucose residue linked in the 1, 4 and 6 positions

might be a product of acid-catalyzed transglucosidation (14,15), and in the second, incomplete oxidation would account for the presence of glucose.

Two further types of anomalous linkage have been reported. Fructose - containing dextrins have been detected in α-amylolytic hydrolysates of waxy maize starch (16), and a significant number of ester phosphate groups (ca. 0.1% phosphorus) have also been found (17).

2. Glycogen

Glycogen resembles amylopectin in many respects, but is more highly branched; it is composed of several thousand α-1,4-linked D-glucopyranose residues which are joined together to give a branched structure (Figure 1). The inter-chain linkages are of the α-1,6-glucosidic type, and usually represent 6-10% of the total, corresponding to an average chain length of 18-10 units.

Bell and Manners (18) have found, by periodate oxidation, that 97-99% of the inter-chain linkages in various glycogens were of the 1,6-glucosidic type. Isomaltose has been isolated from partial acid hydrolysates of glycogen by Wolfrom and Thomson (19), indicating that the inter-chain links have an α-configuration. Some of the α-1,6-linkages may occur in adjacent positions in the molecule. In addition, these workers have detected traces of nigerose (0.002%) in partial acid hydrolysates and claim the presence

of α-1,3-linkages.

Maltulose has been obtained in ca. 5% yield from the salivary α-amylolysis of rabbit liver glycogen (20), which may indicate the presence of fructose as a minor component. Pantlitschko and Matulo have reported the presence of uronic acid and phosphate ester groups in glycogen samples (21), the respective proportions being one group and four groups per five hundred glucose residues. It seems doubtful that such small amounts of uronic acid can be detected, especially with Tollen's naphthoresorsinol reagent which is non-specific for hexuronic acids, giving a reaction with ketoses and pentoses as well. The error involved in the uronic acid estimation used is such that the results quoted must be regarded as inconclusive.

The Fine Structure of Amylopectin and Glycogen

Various arrangements of the constituent chains in the amylopectin and glycogen molecules have been proposed in attempts to explain the chemical and physical properties of these polysaccharides. The first of these was the laminated structure postulated in 1937 by Haworth, Hirst and Isherwood (22). In the same year 'comb' type structures were suggested by Staudinger and Husemann (23). These structures are now no longer accepted in view of more recent experimental evidence.

In 1940 Meyer put forward a multiply-branched "tree" type

structure (Figure 1) based on the results of methylation analyses of the polysaccharides and their β -amylase limit dextrins (24). In such a structure three types of chain may be described (25): A-chain (side-chain) attached to the rest of the molecule only by a 1→6 linkage; B-chain (main chain) to which are attached one or more chains and which is itself joined to an adjacent chain by a 1→6-linkage, C-chain terminated by the single aldehydic group. A-chains along with those parts of B- and C-chains between a branch point and a non-reducing end group are termed exterior chains. Interior chains are those which lie between 1,6-branch points.

Evidence for multiple branching has been obtained by Larner and co-workers (26,27) who subjected amylopectin and glycogen to the successive action of muscle phosphorylase and amylo-1,6-glucosidase. The action of these enzymes which is described in detail on p.17, 21, yielded a series of dextrins, from which successive tiers of branch points had been removed. Additional evidence for multiple branching in amylopectin has been provided by the action of R-enzyme (which hydrolyses the outermost 1,6-linkages) on the β -amylolysis limit-dextrin of waxy maize starch (25,28). The yield of maltose and maltotriose from the outer chain stubs (12.8%) is in accord with a tree-type structure.

Liddle and Manners (29) have found no significant difference in the degree of multiple branching in a number of glycogen and

amylopectin samples. However, similarity in branching characteristics does not control molecular shape since the two polysaccharides show marked differences in hydrodynamic behaviour; e.g. glycogen has a limiting viscosity ca. one tenth that of amylopectin and the relationships between sedimentation coefficient and concentration are different. Since glycogen has very short interior chains the molecule will be very compact and is probably elliptical in shape; amylopectin, although less compact, has been reported to be similar in shape and to have a molecular weight considerably greater than that of glycogen (30).

Banks and Greenwood (30) have recently fractionated glycogen and amylopectin and obtained fractions with the same radius of gyration. Light scattering measurements showed that both polysaccharides were the same shape but the glycogen fraction had a molecular weight of 350×10^6 while that of amylopectin was 50×10^6. The molecules of glycogen are therefore much more compact than those of amylopectin which tend to have a loose structure. The difference in viscosity between the two polysaccharides is probably due to the loose amylopectin molecules becoming entangled with one another under the conditions of viscosity measurement, (0.5%) concentration of amylopectin) in which the polysaccharide molecules, which are very large, will be closely packed.

B. Enzymic Synthesis and Degradation of α -1,4-Glucosans

The majority of enzymes involved in the synthesis and degradation of starch and glycogen are transglycosylases, that is, they catalyze reactions of the type:-

$$D - OR + H - OA \rightleftharpoons D-OA + H - OR$$

where D is a glucosyl residue, R and A may also be carbohydrate residues. H-OA is referred to as the acceptor substrate. The reaction may proceed via an intermediary glucosyl-enzyme complex. Barker and Bourne (31) have suggested the following mechanism:-

$$D - OR + \text{Enzyme} - H \rightleftharpoons \text{Enzyme} - D + H - OR \quad \ldots\ (1)$$

$$\text{Enzyme} - D + H - OA \rightleftharpoons D-OA + \text{Enzyme} - H \quad \ldots\ (2)$$

This mechanism has two stages, the first of which involves a reaction between the donor and enzyme to form the glucosyl-enzyme complex (equation 1) which then reacts with the acceptor substrate with transfer of the glucosyl moiety, (equation 2).

An alternative mechanism has been proposed by Jermyn (32) as follows:-

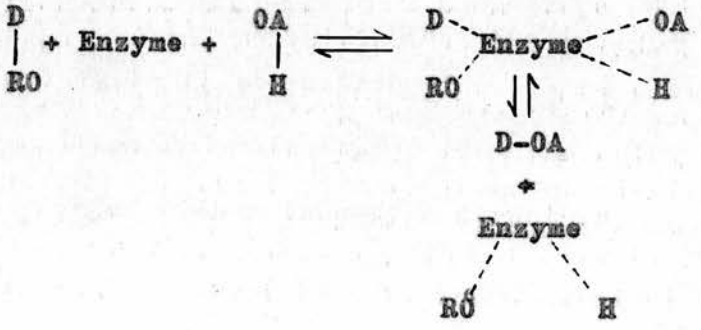
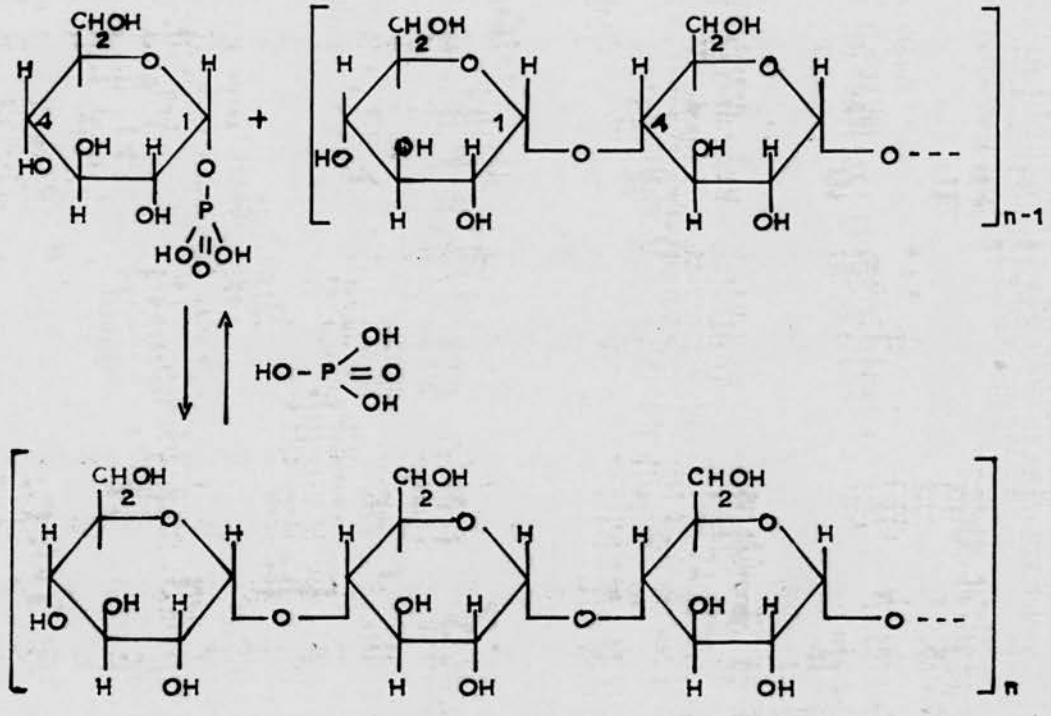


FIGURE 2



In this mechanism the donor and the acceptor substrates combine with the enzyme to form an intermediate complex. Dissociation of this complex completes the reaction with formation of the product.

In both mechanisms the formation of the intermediate complex results in an activation of the substrate and hence a lowering of the activation energy required for the second phase of the reaction.

1. Starch Synthesizing Enzymes

(a) Plant Phosphorylase (P-Enzyme)

This enzyme which was first discovered in peas and potatoes by Hanes (33,34), has been prepared in crystalline form (35,36). It catalyzes the reaction shown opposite (Figure 2) which is freely reversible, the equilibrium depending on the ratio of glucose -1-phosphate to inorganic phosphate.

The above mechanism only proceeds in presence of a primer, the smallest being maltotriose (37). Maltotetraose and higher maltodextrins, amylose, amylopectin and glycogen are more efficient primers than maltotriose. In all cases glucose residues are attached to the non-reducing end-groups of the acceptor (or primer) chains by α-1,4-linkages.

Synthetic amyloses have been prepared by the action of this

enzyme and glucose -1- phosphate in the presence of a primer e.g. maltohexaose. The synthetic action is a random one (i.e. multi-chain action) so that all primer molecules tend to increase uniformly in length (38). P-enzyme may therefore be used to prepare amyloses of known DP.

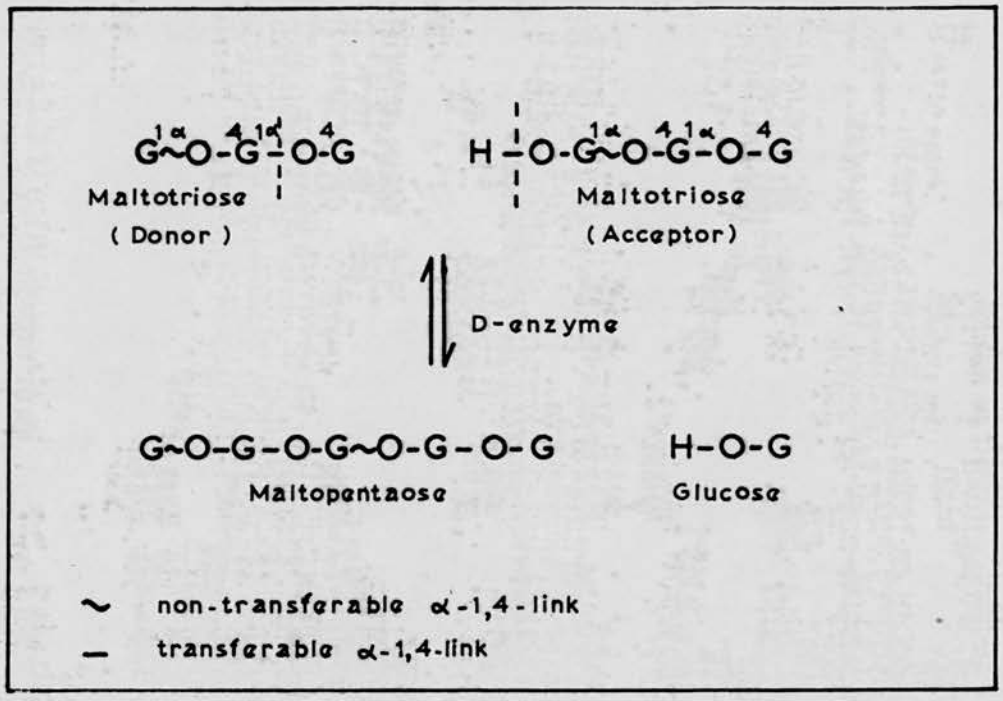
(b) Q-Enzyme

Haworth, Peat and Bourne (1944) isolated an enzyme from potato juice which on incubation with P-enzyme and glucose-1-phosphate produced an amylopectin-type polysaccharide (39). The enzyme, which was called Q-enzyme, also catalyzed the conversion of amylose into amylopectin (40).

Q-enzyme removes a portion of the amylose-type chain which must have a DP between 42-116 glucose residues (41,42) and rejoins it to an acceptor molecule by an α-1,6-linkage between the aldehydic-group of the severed portion and a primary hydroxyl-group in the acceptor. Acceptors can be either amylose-type chains or branched molecules. Q-enzyme may also be able to rearrange 1,6-linkages within a particular amylopectin molecule but there is no evidence that its action is reversible (i.e. the conversion of α-1,6 linkages to α-1,4-linkages).

The extended action of purified Q-enzyme on potato amylopectin has been studied by Peat, Turvey and Jones (1959) who found practically no change in the degree of branching or iodine

FIGURE 3


Maltotriose
(Donor)
Maltotriose
(Acceptor)
D-enzyme
Maltopentaose
Glucose
~ non-transferable α-1,4-link
— transferable α-1,4-link

staining power after a 60 hr. incubation period (43). They concluded that Q-enzyme has no transferase action on natural amylopectin.

Pazur, Budovitch and Tipton (44) have suggested that Q-enzyme may also be responsible for the presence of α-1,3-linkages in amylopectin, although there is no direct experimental evidence on this point.

(c) D-Enzyme

D-enzyme was first discovered in potato juice by Peat, Whelan and Rees (45) and is also present in the broad bean (46). This enzyme catalyzes the transfer of segments from a donor to an acceptor molecule by breaking and making an α-1,4-linkage. The smallest donor is maltotriose; higher maltodextrins, amylose and amylopectin may also be donors. These carbohydrates can all act as acceptors (Figure 3), but the smallest acceptor is glucose. Maltose is not a product of D-enzyme action (47) as there are certain linkages in maltodextrins on which it cannot act, e.g. the linkage at the non-reducing end-group and that penultimate to the reducing end-group (48).

D-enzyme does not change the total number of linkages or average DP. It was first detected by its ability to produce iodine staining material from achroic maltodextrins and has been used to synthesize amylose (49). It has been suggested (50) that the function

of the enzyme is two-fold:-

1. To supply primer for P-enzyme action.
2. To synthesize amylopectin in conjunction with Q-enzyme.

(d) T-Enzyme

Abdullah and Whelan (51) have recently discovered a transglucosylase in the potato (T-enzyme) which has a two-fold action a) the conversion of α-1,4-linkages to α-1,6-linkages, and b) the redistribution of α-1,6-linkages. Typical reactions are a) the formation of panose from maltose and b) the synthesis of isomaltotriose from isomaltose. Enzymes of the type which catalyze conversion a) have been found in extracts from moulds (52), algae (53) and protozoa (54).

2. In vivo Degradation of Starch

(a) P-Enzyme

In the presence of inorganic phosphate, P-enzyme catalyzes the phosphorolysis of natural amylose and amylopectin giving respectively about 70% and 40% degradation to glucose-1-phosphate, since enzyme action ceases at anomalous links or branch points. A detailed account of the phosphorolysis of starch and glycogen will be given in section 6.

(b) R-Enzyme

The α-1,6-linkages in amylopectin or a P-enzyme limit-

dextrin or β-amylolysis limit-dextrin can be hydrolysed by an enzyme (R-enzyme) isolated from the potato and broad bean by Hobson, Whelan and Peat (55). R-enzyme has no action on amylose, isomaltose, dextrin or glycogen of normal structure (56) (i.e. average chain length (CL) ca.12) although it has a limited action on glycogen of CL 18 (57). The relative proximity of the α-1,6-linkages in glycogen may sterically hinder the enzyme action. If glycogen is pre-incubated with α-amylase, which causes random hydrolysis of alternate α-1,4-linkages, the branch points are made accessible to R-enzyme action.

R-enzyme, as prepared by Hobson, Whelan and Peat (55), has recently been separated into two active fractions by MacWilliam (58). The first of these can debranch amylopectin but has no action on α-limit dextrins, while the second can attack only these limit dextrins. The latter fraction resembles the enzyme "oligo-1,6-glucosidase" reported by Larner and McNickle (59) to catalyze the hydrolysis of α-1,6-linkages in isomaltose, panose and α-limit dextrins.

The smallest substrate for R-enzyme action is 6^3- α-maltosylmaltotriose, this being the smallest branched product of the salivary α-amylolysis, at low enzyme concentration, of amylopectin and glycogen (11).

(c) D-Enzyme

D-enzyme action is freely reversible and in the presence of excess glucose, as acceptor, it will disproportionate amylose type chains giving products of lower DP. The enzyme is also capable of shortening the exterior chains of amylopectin and will presumably act on those of glycogen also.

3. Degradation of Starch-Type Polysaccharides by Hydrolytic Enzymes

(a) β-Amylase

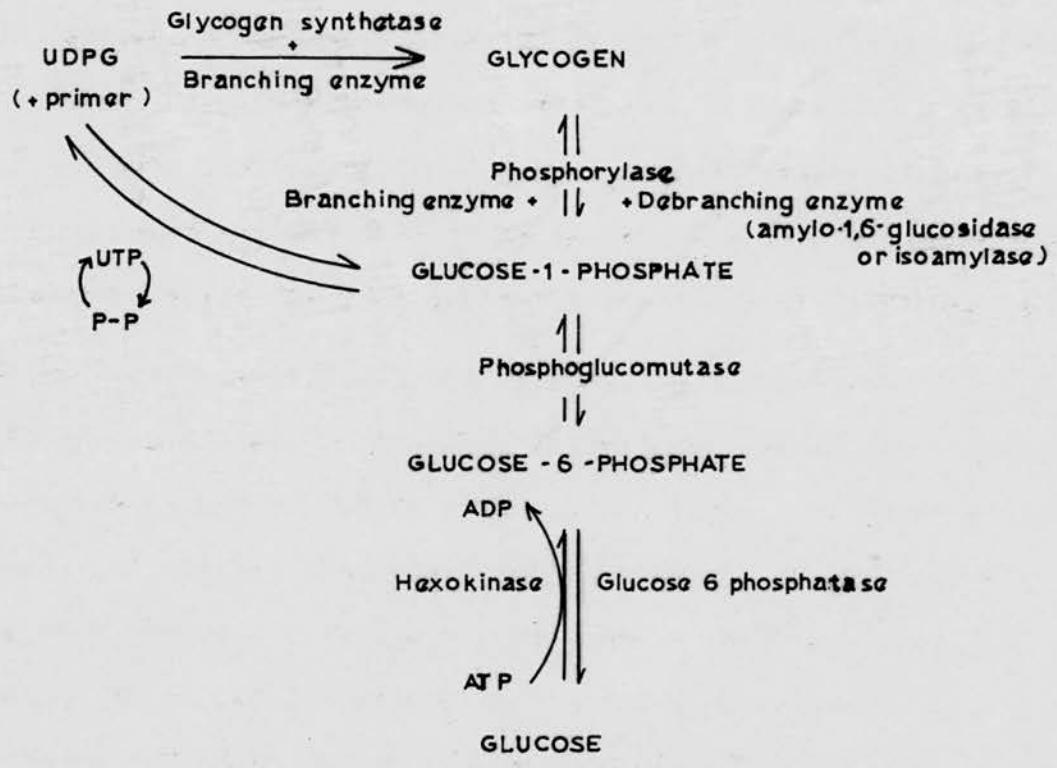
This enzyme is found in the seeds and other parts of the higher plants; in the cereals the enzyme is apparently in highest concentration before germination. Those from soya bean (2), barley (60), wheat (61) and sweet potatoes (62) have been highly purified.

β-amylase catalyzes the stepwise hydrolysis of alternate linkages in chains of α-1,4-linked glucose residues, starting from the non-reducing ends and producing β-maltose. The action is confined to the exterior chains of amylopectin and glycogen producing β-maltose and a limit dextrin with exterior chain stubs which are 2-3 glucose units in length (25,63). Linear amyloses are completely degraded by β-amylase.

(b) α-Amylases

The linkage specificity of the α-amylases is the same

FIGURE 4



in vivo SYNTHESIS OF GLYCOGEN FROM GLUCOSE

as for the β-amylases, but the former catalyze a random hydrolysis of α-1,4-glucosidic-linkages producing α-maltose as the major product. The α-amylases can by-pass α-1,6-linkages and thus degrade the interior parts of amylopectin and glycogen giving maltose and a series of branched α-limit dextrins. In the case of salivary α-amylase the smallest of these is 6^3-α-glucosylmaltotriose (64). Amyloses are completely degraded by α-amylase giving maltose and maltotriose in the molar proportions 2.4:1 or maltose and glucose depending upon the enzyme concentrations.

α-amylases are widely distributed among animals and higher plants; many moulds and bacteria produce α-amylase. In starch-bearing plants, α-amylase is mainly formed during germination. A wide variety of α-amylases from different sources have been highly purified. The exact specificity of α-amylase for 1,4-linkages near a 1,6-inter-chain linkage and such properties as pH optimum and specific activation and inhibition varies according to the source (65).

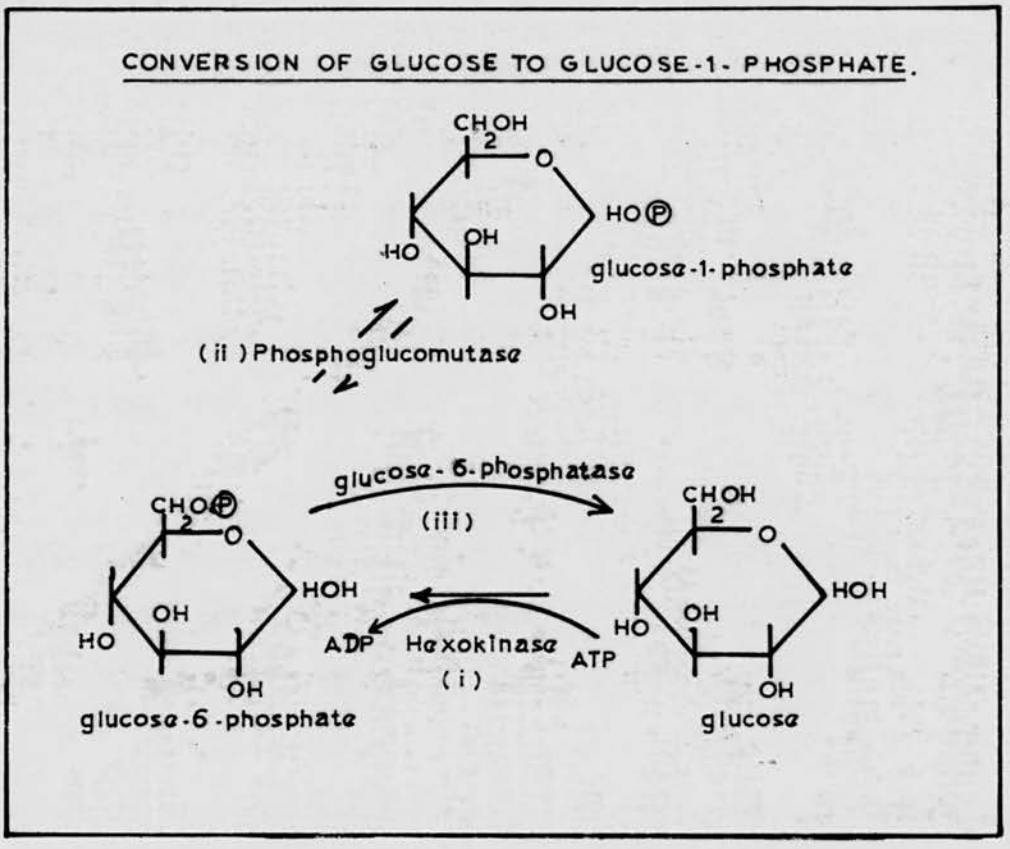
A detailed account of α-amylases will be given in Section 5.

4. Synthesis and Degradation of Glycogen.

Glycogenesis - Glycogenolysis Relationship

Glycogenesis is the build up of glycogen from carbohydrate material (usually glucose); the process of breakdown to glucose is termed glycogenolysis (Figure 4).

FIGURE 5


CONVERSION OF GLUCOSE TO GLUCOSE-1- PHOSPHATE.
CH2OH
O
HO Ⓟ
HO
OH
OH
glucose-1-phosphate
(ii) Phosphoglucomutase
glucose-6-phosphatase
(iii)
CH2OⓅ
HOH
HO
OH
OH
glucose-6-phosphate
ADP
Hexokinase
(i)
ATP
CH2OH
HOH
HO
OH
OH
glucose

In this discussion the term glycogen will be taken to include both the reserve polysaccharide of animal tissues and the structurally related branched α-1,4-glucosans in certain yeasts (66,67), bacteria (68) and protozoa (69).

The glycogenesis - glycogenolysis process involves a complex enzyme system, which resembles that involved in the synthesis and degradation of starch. The individual enzymes of the animal and plant systems have similar modes of action, although they differ in chemical constitution and other respects.

(a) Interconversion of Glucose and Glucose-1-Phosphate

Three enzymes are involved in the reversible transformation of glucose to glucose-1-phosphate which is represented in Figure 5 on the opposite page.

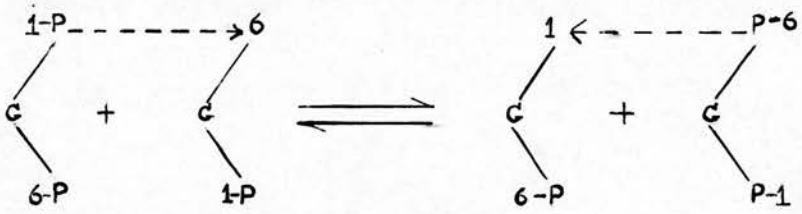
(i) Hexokinase

Glucose is phosphorylated to glucose-6-phosphate at the expense of adenosine triphosphate (ATP) under the influence of the enzyme hexokinase.

The energy content of the pyrophophate bond of ATP is equivalent to about 12,000 calories per mole, the ester phosphate to only 3,000 calories per mole. Owing to the large decrease in free energy, the equilibrium of the reaction is far to the side of glucose-6-phosphate, and a different enzyme (glucose-6-phosphatase) is needed to catalyze the reverse transformation.

(ii) Phosphoglucomutase

This enzyme catalyzes the reverse transfer of phosphate from C.6 to C.1 of glucose. Leloir (70) has shown that the shift of the phosphate involves glucose-1,6-diphosphate in the following type of reaction:



(iii) Glucose-6-Phosphatase

The hydrolysis of glucose-6-phosphate is catalyzed by a specific phosphatase, which acts at a neutral pH unlike the less specific alkaline and acid phosphatases. The glucose-6-phosphatase is present in high concentration in the liver, which is responsible for the formation of blood sugar, and in lower concentration in the kidney. The enzyme occurs in very low concentration in muscle which contributes no sugar to the blood.

(b) Phosphorylase

These enzymes catalyze the same reaction as the plant P-enzyme. Phosphorylase was first isolated from muscle by Cori, Schmidt and Cori (71), from liver by Ostern and Holmes (72) and from yeast by Schaffner and Specht (73). Cori and Green (74) found that tissue phosphorylases required a co-enzyme, adenylic acid, but it was shown later that this was not needed by the yeast

enzyme (75).

Muscle phosphorylase exists in at least two forms, phosphorylase 'a' which is active in the absence of adenylic acid and phosphorylase 'b', inactive in the absence of this nucleotide. The two forms of the enzyme are interconvertible, the 'b' form being converted to the 'a' form by phosphorylase kinase, and the reversal of this process being brought about by a de-activating enzyme, phosphorylase phosphatase (PR enzyme). Phosphorylase 'b' has half the molecular weight of phosphorylase 'a'.

Pyridoxal-5-phosphate is the prosthetic groups of phosphorylase (76), four moles being present per mole of phosphorylase 'a'. No direct participation of the phosphate groups of pyridoxal-5-phosphate in the mechanism of the phosphorylase reaction has yet been shown (77).

Cori and Cori (78) have shown that a primer is necessary for the synthetic reaction. Although glycogen acts as a primer, amylose-type chains with the same DP as the unit-chains in glycogen do not prime the reaction (79). Amylose type chains ($DP \leqslant 200$) and amylopectin also act as primers. In contrast P-enzyme can utilize maltotetraose as an efficient primer.

Phosphorylases from animal tissues cause partial degradation of glycogen, in presence of inorganic phosphate, limits varying from 28-49% (80). Amylose and amylopectin are also degraded, the limits

being approximately 70% (81) and 36-57% (82) conversion into glucose-1-phosphate, respectively. Phosphorolysis is limited to the exterior chains of glycogen and amylopectin as the enzyme cannot by-pass α-1,6-linkages. From an examination of the muscle phosphorylase (ϕ) limit-dextrins of glycogen and amylopectin, Cori and Larner (27) concluded that the A-chain stubs were single α-glucose units joined through 1,6-linkages to the B-chains which were 6-7 units long. More recent evidence has shown that the A-chains and outer B-chain stubs in these phosphorylase-limit dextrins are each 4 glucose units long (83).

The activating and de-activating enzymes, phosphorylase kinase and PR enzyme respectively control phosphorolysis. Any factor known to activate phosphorylase (b form $\longrightarrow$ a form) always leads to increased glycogenolysis rather than synthesis (84,85).

(c) Uridine Diphosphate Glucose (UDPG) Transferase or Glycogen Synthetase.

A second enzyme system in addition to phosphorylase, capable of synthesizing α-1,4-glucosidic linkages has been found recently in liver (86) and muscle (87) extracts. The action is two-fold, firstly the formation of uridine diphosphate glucose and secondly the transfer of the glucose to the terminal units of pre-existing glycogen molecules, the new linkage being of the α - 1,4-type:-

UDPG Pyrophosphorylase

UTP + G - 1-P $\rightleftharpoons$ UDPG + P-P ..... (1)

UDPG Transferase

UTP + glycogen $\longrightarrow$ (glycogen + 1) + UDP ..... (2)

(UTP, uridine triphosphate; G-1-P, glucose-1-phosphate; UDPG, uridine diphosphoglucose; P-P, pyrophosphate; UDP, uridine diphosphate).

Reaction 1 is catalyzed by a pyrophosphorylase, reaction 2 by the uridine diphosphate glucose transferase or glycogen synthetase. The enzyme action is not primed by either maltoheptaose or a mixed cellodextrin fraction (88). Such a system would probably act with branching enzyme otherwise the resulting glycogens would have very long exterior chains. The reversibility of reaction 2 is doubtful, in vitro experiments showing that the system favours glycogen synthesis rather than breakdown, under physiological conditions (89). Further evidence for the non-reversibility of the reaction was found by Mommaerts and co-workers (90) who reported a case of glycogen storage disease in which the enzyme lesion was phosphorylase. Continued deposition of glycogen in the presence of the UDPG transferase system suggests a balance to the side of glycogen synthesis.

(d) Branching Enzymes

(i) Animal Branching Enzyme

The α-1,6-branch points in glycogen are synthesized by an amylo-(1,4 $\longrightarrow$ 1,6)-transglucosidase (branching enzyme) discovered in

rabbit heart and muscle extracts (91), which breaks a small number of α-1,4-linkages in an amylose-type chain and synthesizes an equal number of α-1,6-linkages. The enzyme will not apparently act on amylose, unlike Q-enzyme, but can act on the outer chains of glycogen (92) ($DP \geqslant 6$) and of amylopectin. Simple sugars such as maltose will not act as acceptors for the transfer of glucose chains.

(ii) Yeast Branching Enzyme

Gunja, Manners and Maung (93) have studied the action of a branching enzyme, found in yeast extracts, on amylose and amylopectin. The respective products, were amylopectin and glycogen-type polysaccharides. Further branching was observed when the 'synthetic' amylopectin was re-incubated with the branching enzyme showing that amylopectin is an intermediate in the branching of amylose, the limiting product being glycogen. The branching enzyme had no action on a horse muscle glycogen of CL 17, but limited action was observed on malted-barley amylopectin, CL 18. This is probably due to the amylopectin having exterior chains of sufficient length to enable branching enzyme to act. The exterior chains of the glycogen would be shorter and resistant to enzyme attack.

(e) Debranching Enzymes

(i) Amylo-1, 6-Glucosidase

Cori and Larner (27) isolated an enzyme from muscle extracts,

amylo-1,6-glucosidase which, acting in conjunction with phosphorylase, caused complete degradation of glycogen. The enzyme prepared by the method of Cori has now been found to contain an α-1,4-glucosyl transferase (83) of the type known to exist in animal tissues (94,95,96). The transferase may correspond to the D-enzyme of plants. Owing to the unusual specificity requirement of amylo-1,6-glucosidase for a single glucose stub, it is likely that glycogen degradation will be incomplete unless both amylo-1,6-glucosidase and the transferase are present. This fact will be considered again in Section 4.

(ii) Isoamylase

The presence of a debranching enzyme in brewer's yeast was first reported by Meyer and Bernfeld (97). The mode of action of this enzyme (isoamylase) has been studied by Maruo and Kobayashi (98) and more recently by Manners and Maung (99). The enzyme will hydrolyse α-1,6-linkages in amylopectin and glycogen and their respective β-limit dextrins but the action is incomplete. Gunja (100) has suggested that isoamylase action may be limited to 1,6-linkages of A-chains and in the case of amylopectin, further hindered by the presence of ester-phosphate groups. Additional evidence indicates that the longer outer chains of amylopectin constitute a hindrance to enzyme action.

The inter-relationship between the glycogen-metabolizing enzymes is shown in Figure 4. In Section 4 it has proved possible

to trace deficiencies of some of these enzymes by analysis of glycogen structure thus getting a better insight into the mode of *in vivo* synthesis and degradation.

5. In vivo Synthesis of Starch

Synthesis of glycogen from α-glucose-1-phosphate is accomplished by the combined action of chain building enzymes (phosphorylase and UDPG transferase) and branching enzymes. Glycogen is a limiting structure with respect to degree of branching, small differences in branching characteristics probably being due to changes in the relative activity of the branching enzyme. The combined action of the corresponding plant enzymes in starch synthesis does not explain the formation of amylose and amylopectin, neither of which are self-limiting structures, at the same site.

One explanation for the concurrent synthesis of the components of starch is that there are two enzyme systems working side by side, one having a "chain builder" plus branching enzymes, the other having enzymes which can only synthesize α-1,4-linkages. Such an explanation has been proposed by Whelan (50) who postulates two compartments in the space to be filled by starch, separated by a semipermeable membrane and capable of allowing through molecules up to the size of maltotetraose which can act as primers for D-enzyme and phosphorylase. D-enzyme, Q-enzyme and presumably T-enzyme are in one compartment while hexokinase (+ ATP), phosphoglucomutase and

phosphorylase, are in the other. The presence of maltotetraose or a higher maltodextrin is needed to prime the reaction. This system gives a resulting weight ratio, amylopectin: amylose, of 3:1 which corresponds to most natural starches. On this hypothesis, waxy cereals should have no membrane system.

Erlander (101) has proposed that glycogen is an intermediate in the formation of starch, the conversion being effected by a debranching enzyme. The debranching enzyme is supposed to be phosphorylated and to transfer its phosphate group to a branch point of the glycogen molecule and to become linked with the reducing group of the branch which it can then transfer to a linear accept or molecule. The result would be a debranched glycogen (amylopectin) and a series of long linear chains (amylose), formed by a rapid action, which would crystallize before branching enzyme attack. A low activity of the debrancher would explain the virtual absence of amylose in the waxy cereal starches.

This hypothesis explains the origin of ester phosphate groups in linear parts of the amylopectin molecule. The necessary "glycogen" intermediate cannot be synthesized by Q-enzyme and phosphorylase alone, but T-enzyme might be capable of converting amylopectin to glycogen.

The debranching enzyme (still to be detected and isolated) catalyzes the reverse reaction to that demonstrated by Gunja, Manners and Maung (93), i.e. the conversion of amylose to glycogen,

via amylopectin. The mechanism proposed by Erlander is unlikely.

It is almost certain that future research will produce new enzymes and perhaps hitherto unknown systems, which will provide a more complete knowledge of the *in vivo* synthesis of starch and glycogen. It will be of interest, for instance, to discover if there is a plant counterpart to the animal UDPG transferase system.

Scope of the Present Work

The present studies have been directed towards increasing our knowledge of the molecular structure and metabolism of glycogen and the starch components, amylose and amylopectin.

For this purpose semi-micro methods of analysis have been examined including (a) end-group assay by periodate oxidation on a 100 mg. rather than 200-300 mg. scale, (b) the interaction with iodine and concanavalin-A, both of which require only milligramme quantities of polysaccharide, and (c), enzymic degradation methods.

Since β-amylase occurs with z-enzyme, attempts have been made to separate the two enzymes, and to study the degradation of the α-1,4-glucosans, especially amylose, by the purified enzymes.

The three polysaccharides have also been degraded with P-enzyme which has enabled the properties of the plant and animal

phosphorylases to be compared, and the fine structure of amylose to be studied.

The possible relationship between the extent of degradation of a polysaccharide by salivary α-amylase and the degree of branching has been investigated as a potential method for determination of average chain length. Investigations into the relationship between the structure and metabolism of glycogen have been carried out. Glycogen from new biological tissues has been examined, and additional properties of these and other glycogens have been measured. The metabolism of ox and horse muscle glycogens during *rigor mortis* and of human glycogens from cases of glycogen storage disease have also been studied.

Section II

GENERAL METHODS

The following general methods have been used throughout this work.

1. Estimation of Reducing Sugars

Glucose and maltose were estimated with the Somogyi (1952) reagent (102), calibrated against known quantities of the respective sugars. Concentrations of standard sugar solutions were determined polarimetrically. The ratio, mg. sugar: titre difference from blank (ml. 0.01 N sodium thiosulphate) was found to be 0.285 for glucose and 0.530 for maltose, these figures remaining constant to within $\pm$ 0.01 for different preparations of the reagents. The copper reagent was kept between 37° and 40° to prevent crystallization.

2. Paper Chromatographic Methods

Paper partition chromatography on Whatman No.1 paper was used for the preliminary separation and identification of carbohydrates. Separation was effected by downward displacement at room temperature.

Solvents and Sprays

Solvent 1. Ethyl acetate - pyridine - water (10:4:3)

Spray 1. Ammoniacal Silver Nitrate.

The following solutions were prepared:-

(I) Acetone (200 ml.) was added to saturated silver nitrate solution (1 ml.) followed by the dropwise addition of water until the precipitate just redissolved.

(II) A 0.5 N solution of sodium hydroxide in aqueous methylated spirits.

(III) Ammonium hydroxide (6 N) or a saturated solution of sodium thiosulphate.

The dried paper was dipped in solution I, allowed to dry, sprayed with solution II, and excess reagent removed by washing in solution III. The paper was finally washed with water and allowed to dry (103).

Spray 2. Aniline oxalate.

The dried paper was sprayed with a saturated solution of aniline oxalate in aqueous ethanol; spots were developed by heating the paper at 130° (104).

3. Drying of Polysaccharide Samples

Prior to estimation of purity, polysaccharide samples were

dried in vacuo, at 60°, over phosphorus pentoxide for several hours.

4. Complete Acid Hydrolysis

Exact concentrations of oligo- and polysaccharides were determined by complete acid hydrolysis and estimation of the liberated monosaccharide. The present work involves only α-linked oligo- and polysaccharides with glucose as the repeating unit.

It was found that amylose and other α-1:4-linked glucose polymers were completely hydrolysed to glucose by heating at 100° with 1.5 N sulphuric acid for 1.5 hr. However, glycogen and amylopectin, containing a small number of α-1,6-glucosidic linkages which are more resistant to acid, were not completely hydrolysed under these conditions. Control experiments indicated that the necessary conditions for these polysaccharides were heating at 100° with 2 N sulphuric acid for 1.5 - 2 hr. No destruction of glucose was observed even after heating with 2 N sulphuric acid for 3 hr. In this work, therefore, complete acid hydrolysis of all oligo- and polysaccharides was effected by heating samples at 100° for 2 hr. with 2 N sulphuric acid. Acid hydrolysates were neutralised to phenol phthalein and then made just acid by the addition of 1 drop 0.2 N sulphuric acid. The reducing sugar was then estimated with the Somogyi reagent. The total oligo- or polysaccharide present was equivalent to 0.9 x mg. glucose.

The monosaccharides present in complete acid hydrolysates were examined by paper chromotography after neutralization with barium carbonate followed by deionisation with Amberlite IR4B and 120 resins, and concentration under vacuum at 40°.

5. Deproteinisation

Enzyme digests were deproteinised, when necessary, by the method of Nelson (105) before the estimation of reducing sugars.

Zinc sulphate hexahydrate (5%) and barium hydroxide solution (ca. 3 N) were prepared and adjusted so that 4.7 - 4.8 ml. baryta neutralized 5 ml. zinc sulphate giving a faint pink colour to phenol phthalein. Zinc sulphate solution was added first to the solution to be deproteinized followed by an equal quantity of barium hydroxide, usually 0.5 to 1 ml. depending on the amount of protein. Samples were removed for estimation after removal of the barium sulphate by centrifugation.

6. Iodine Staining

(a) Examination of Spectra in Aqueous Solution

Glycogen or amylopectin solution containing 2.5 mg. polysaccharide was introduced into a 25 ml. standard flask with one drop 6 N hydrochloric acid and iodine solution (2.5 ml; 0.2% iodine in 2% potassium iodide) and the mixture diluted to 25 ml. The extinction

(E) of the resulting polysaccharide-iodine complex was examined in a Unicam SP 600 Spectrophotometer over the range 420-680 mμ against an iodine-water blank, in 1 cm. glass or quartz cells. The wavelength of maximum absorption (λ max.) was taken as the mid-point of the peak obtained by plotting wavelength against optical density. The optical density at λ max. is termed the E max.

(b) Examination of Spectra in Ammonium Sulphate Solution

It was found that if the above conditions for glycogen and amylopectin were used in the presence of half-saturated ammonium sulphate fairly rapid precipitation of the polysaccharide - iodine complex occurred. In addition, the E values were towards the maximum of the Unicam scale which reduced the sensitivity. It was therefore decided to use a lower polysaccharide concentration. To the standard flask (25 ml.) containing glycogen or amylopectin solution (3-5 ml.; 1.25 mg.) and 1 drop 6 N hydrochloric acid, was added saturated ammonium sulphate solution (12.5 ml.) followed by iodine solution (2.5 ml.). After dilution to 25 ml. the polysaccharide-iodine complex was examined as before against an ammonium sulphate-iodine blank.

(c) Blue Value

Amylose and amylopectin were studied under 'blue-value' conditions (106):-

Polysaccharide (15-20 mg.) was dissolved in 0.2 M potassium hydroxide, the solution neutralised, and made up to 20 ml. The

exact concentration was determined by acid hydrolysis of a portion and estimation of the glucose produced. An aliquot containing 5 mg. polysaccharide was transferred to a 500 ml. standard flask and stained with iodine solution (5 ml.). The coloured solution was diluted to 500 ml. after addition of 0.25 ml. 6 N hydrochloric acid. The spectrum was examined between 450 and 750 mμ against an iodine-water blank. The blue value (B.V.) is defined as the E at 680 mμ using 4 cm. cells or the E x 4 using 1 cm. cells.

7. Periodate Oxidation

Determination of Periodate Reduction

The periodate reduction during oxidation with sodium meta-periodate was determined by the method of Fleury and Lange (107) in which the periodate sample was first neutralised with solid sodium bicarbonate followed by the addition of an excess of standard arsenite (ca. 0.1 N) and excess of potassium iodide. After 10-15 minutes at room temperature, the excess arsenite was then titrated with standard iodine solution (0.102 0N).

Determination of Formic Acid

The formic acid released during sodium and potassium periodate oxidation of carbohydrates was estimated by titration of samples, after destruction of excess periodate with ethylene glycol, with approximately 0.01 N standard sodium hydroxide

(carbonate free) using methyl red indicator or a Pye Universal pH meter to an end point at pH 5.8. Nitrogen was bubbled through the solutions during titration to prevent uptake of atmospheric carbon dioxide.

8. α-Amylolysis

The salivary α-amylase used in this work was prepared by A.M. Liddle (1956) by the method of Fischer and Stein (108). The digest conditions used are as follows:-

Polysaccharide solution (10 ml.; ca. 5 mg/ml.) was added to a standard flask (100 ml.) containing α-amylase solution (1 ml.: 5.5 mg/ml. ca. 204 units) and 0.5% sodium chloride (10 ml.). The digest was diluted to 100 ml. and incubated at 37°. Aliquots (5 ml.) were removed after suitable time intervals for estimation of the reducing sugar which was expressed in terms of 'apparent maltose'. The degree of α-amylolysis is defined as the apparent percentage conversion into maltose (P_M value).

9. β-Amylolysis

A commercial sample of β-amylase purchased from the Wallerstein Laboratories (New York) was used in part of this work. The enzyme was free from maltase but contained a trace of Z-enzyme (α-amylase).

The activity of the preparation was 110 units/mg. determined by the method of Hobson, Whelan and Peat (109).

Digests, with a final polysaccharide concentration of ca. 1 mg/ml., were prepared in the following way:-

(a) β-Amylolysis of Glycogens

Glycogen (10-30 mg.) was incubated at 37° in 0.04 M acetate buffer, pH 4.6 with β-amylase (50 units/mg.). Aliquots (2-3 ml.) were removed after 24 and 48 hr. for estimation of the maltose produced, enzymic action being expressed as the percentage conversion into maltose. Digests incubated at pH 3.6, when Z-enzyme is inhibited, gave identical results to those obtained at pH 4.6. Inhibition of Z-enzyme was therefore unnecessary for experiments with glycogen.

(b) β-Amylolysis of Amylopectins

Amylopectin (10-30 mg.) was incubated under the above conditions except that the enzyme concentration was decreased to 25 units/mg. polysaccharide since amylopectin has fewer non-reducing end groups (ca. one half the number of the same weight of glycogen). Enzymic action was complete after 24 hr., the β-amylolysis limit for this sample being quoted.

Results obtained at pH 3.6 under the same conditions gave the same result. When the enzyme concentration was increased to 50 units/mg. or more at pH 4.6 a gradual increase of maltose

production was observed, due to Z-enzyme action. The enzyme concentration was therefore maintained below this level, i.e. at 25 units/mg. or less, so that a true β-amylolysis limit could be obtained.

(c) Amylose

Amylose (10-30 mg.) was incubated with 0.04 M acetate buffer at pH 3.6 and 4.6 with β-amylase (100 units/mg. at pH 3.6; 50 units/mg. at pH 4.6) and the digests analysed as above. At pH 3.6, β-amylase was pre-incubated with the buffer for 0.5 hr. at room temperature before addition to the polysaccharide.

Toluene (1-2 drops) was added to all enzyme digests as an antiseptic. Polysaccharide concentrations were determined by acid hydrolysis of aliquots removed from the digests themselves, or the polysaccharide solution before addition to the digests. The former method was used when there was no interference by the enzyme in the glucose estimation.

10. Estimation of Phosphate

The method used was that of Allen (110). The following reagents were prepared:-

Perchloric acid. A 60% solution was prepared.

Amidol. Sodium metabisulphite (10 g.) was dissolved in distilled water (ca. 45 ml.). Amidol (0.5 g.) was added and

after thorough mixing, the solution was filtered and made up to 50 ml. The solution was stored at 2° and reprepared every 2 weeks when in use.

Ammonium Molybdate. A 10% solution of ammonium molybdate was prepared, a few ml. concentrated ammonia being added to facilitate solution.

An aliquot of solution containing inorganic phosphate (0.01-0.15 mg.) was placed in a 25 ml. standard flask and diluted to 19 ml. Perchloric acid (2 ml.), amidol (1 ml.) and ammonium molybdate (1 ml.) were added in that order, thorough mixing being ensured after each addition. After dilution, the blue colour was read after 5 minutes against a reagent blank on an EEL colorimeter with a red filter (680 mμ ., No.608).

Standard potassium dihydrogen phosphate solution was used to calibrate the above reagents. The calibration curve was reproducible.

Whelan and Bailey (38) suggested the use of 1 ml. amidol instead of the 2 ml. of reagent in the original method, as more stable solutions are obtained.

Glucose-1-Phosphate in Presence of Inorganic Phosphate. An aliquot (2 ml.) containing glucose-1-phosphate (ca. 0.40 - 6.30 mg.) and inorganic phosphate (0.1 M) was diluted with distilled water (2 ml.) in a 25 ml. standard flask. Magnesia mixture (10 ml.;

8.6 g. $MgCl_2.6H_2O$ 13.50 g. NH_4Cl and 35 ml. concentrated ammonia dissolved in 1000 ml.) was added at 40-60° and the mixture allowed to stand at room temperature for 30-60 minutes. After making up to the mark and filtering, aliquots (5 ml.) of the solution were hydrolysed at 100° for 7 minutes with concentrated hydrochloric acid (0.65 ml.) in 25 ml. standard flasks. The phosphate contents in the hydrolysed and unhydrolysed samples were measured, the difference giving the total phosphate liberated from the glucose-1-phosphate. Control experiments have shown that under the above conditions no glucose-1-phosphate is adsorbed on to the magnesium ammonium phosphate precipitate.

11. Glycogen Value

A solution of the globulin Concanavalin A was prepared for the determination of glycogen values, by the method of Cifonelli and Smith (111).

Preparation. Jack bean meal (20 g.) was stirred with sodium chloride (2%; 200 ml.) for 10 minutes and centrifuged (10 min.; 3000r.p.m.). Acetate buffer pH 4.2 (2 ml.; 2.0 M) was added to the turbid supernatant solution and after 1 hr. this was centrifuged at room temperature. The supernatant solution was heated to 55° with stirring, allowed to stand for 0.5 hr. at room temperature, then centrifuged. If at this stage the solution was still turbid, glycogen solution (0.1%; 2-3 ml.) was added and the mixture was left for ca. 24 hr. at 2°. The solution obtained

after centrifugation was then reasonably clear and this was stabilized by addition of poly vinyl alcohol (7%; 15 ml.) and stored at 0°. This stock solution was centrifuged before use since it tended to become turbid on standing.

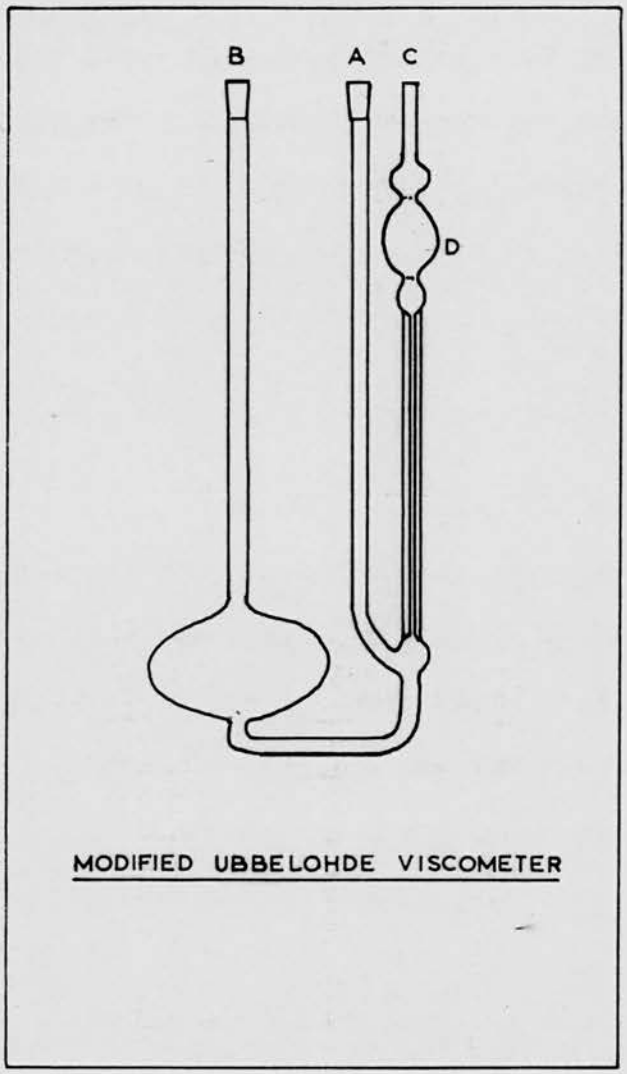
Measurement of Glycogen Value (G.V.). Glycogen solution (1 ml.) containing ca. 800 - 1000 μg. polysaccharide, was added to concanavalin A solution (9 ml.) and after 10-15 minutes the extinction at 420 mμ was measured on a Unicam SP 600 against a reagent-water blank. Values of extinction were measured at three concentrations of each glycogen (0-1 mg.). The extinction value for 1000 μg. was calculated from a graph of extinction versus concentration. This value was compared with that of a standard sample of rabbit liver glycogen (G.V.1.00) provided by Professor F. Smith (112).

It was necessary to use a standard of known G.V. as a control in each determination.

12. Viscosity Determination. The specific viscosity (η_{sp}) of a polymer solution is defined as the relative increase in viscosity:-

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} = \frac{\eta}{\eta_o} - 1 = \eta_r - 1$$

where η is the solution viscosity, η_o is the solvent viscosity and η_r, the relative viscosity is the ratio $\frac{\eta}{\eta_o}$.



MODIFIED UBBELOHDE VISCOMETER

FIGURE 6

η_{sp} is concentration dependant, the ratio $\frac{\eta_{sp}}{c}$ being termed the viscosity number. The limiting viscosity number or intrinsic viscosity $[\eta]$ is the value obtained by extrapolation of $\frac{\eta_{sp}}{c}$ to infinite dilution

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c}$$

where c = concentration in g/ml.

For the determination of η_{sp} and $[\eta]$ a modified Ubbelohde viscometer was used. The expression for viscosity in viscometers of this type involves a kinetic energy correction which can be ignored when a long flow time is used (t $>$200 sec.). The expression for the specific viscosity, in dilute solution, is given by:-

$$\eta_{sp} = \frac{T}{T_o} - 1$$

where T is the solution flow time and To is the solvent flow time, in seconds.

Measurements were made in a constant temperature bath at 25° with the viscometer clamped in a vertical position. Solutions were filtered through G4 sintered glass before use. All additions to the viscometer (Figure 6) were made down tube A, and solutions were mixed by blowing dust-free air (through G1 sintered glass) gently down A. Flow times were measured by sealing A and exerting pressure at the top of B, thus forcing some liquid up the capillary (0.5 mm.) and into the bulb at the top of C. The flow time was

the time taken for the liquid level to pass two marks, one above and one below bulb D. Times were measured with a stop watch to the nearest 0.1 sec. Two methods were used for the determination of viscosities:-

(1) Solution (10-15 ml.) was placed in the viscometer and the flow time determined. Solvent (5 ml.) was added and the flow time redetermined after each addition. The solvent flow time was measured separately.

(2) Solvent (10 ml.) was placed in the viscometer and the flow time determined. Aliquots (3-5 ml.) of solution were added and the flow times measured. This latter method was preferred to the former as the solvent flow times could be determined without removing the viscometer from the water bath.

Concentrations were always measured by estimation of the glucose obtained by complete acid hydrolysis of an aliquot from the filtered solution, or from the viscometer after the final dilution.

For amyloses of high molecular weight it has been shown (113) that the following relationship holds: $DP = 7.4 [\eta]$.

SECTION 3

Studies on the Branching Characteristics of Glycogen

Introduction

Glycogen is distributed throughout the cell cytoplasm, is cold water soluble after extraction, and gives a characteristic red-brown stain with iodine which distinguishes it from amylopectin. A portion of the glycogen in the cell is water soluble and a part is insoluble, possibly because it is bound to protein by either covalent linkages, physical entanglement, or hydrogen bonding. Muscle glycogen is closely associated with protein.

The solvents normally used for the extraction of glycogen from animal tissue are hot water, cold aqueous trichloroacetic acid (TCA), and hot 30% potassium hydroxide. The majority of glycogens reported in the literature have been extracted by either the first or the last methods these giving glycogens of comparable molecular weight in the range 2-10 x 10^6. Stetten, Katzen and Stetten (114) have reported molecular weights as high as a 100 x 10^6 (from light scattering measurements) for TCA extracted glycogens which, they claim, are more representative of native glycogen. Pollard (115) has suggested that in the largest glycogen molecules, some of the bonds near the reducing-end of the molecule will be under strain due to a packing of groups (glucose residues) on the surface, and that on

thermal agitation these might break to relieve the strain, thus causing a reduction in molecular weight.

Cori and Cori (116) postulate that the high molecular weight values of TCA glycogens are due to molecular aggregates held together by traces of protein. That proteins and polysaccharides can form aggregates has been demonstrated many times. Madsen and Cori (117) have calculated a limiting size for the glycogen molecule of ca. 22×10^6, based on calculations of the available surface area for the end-group glucose residues. If glycogen were to exceed this size it would be necessary for some of these end-groups to terminate within the molecule.

Extraction with hot 30% potassium hydroxide has been criticized as causing considerable degradation (118). It has been shown (114, 119) that hot dilute alkali in presence of oxygen has a considerable degradative effect but with hot concentrated alkali there is no progressive reduction in molecular weight. Staudinger (120) observed no decrease in molecular weight (light scattering) of two glycogen samples after heating in 30% potassium hydroxide for 1 hr. at 100°. Bryce, Greenwood and Jones (118) found no decrease in sedimentation constant of rabbit liver glycogen after similar treatment for 3 hrs. The difference in degradative action of the two methods may be due to the low solubility of oxygen in the latter. Hot 30% potassium hydroxide does not therefore seem to cause appreciable degradation of the glucopyranosidic linkages in a glycogen molecule.

In this Section a study has been made of the branching

characteristics of a number of glycogens extracted with either hot water or 30% potassium hydroxide. The molecules of the glycogens under consideration may be the result of slight degradation of larger molecules, or they may represent the 'native' polysaccharide. Even if the former statement is correct it is probable that the observed branching characteristics are representative of the 'native' glycogen, as a series of molecular weight fractions of liver glycogen having similar branching characteristics has been isolated by Stetten and Stetten (121).

Periodate oxidation results in conjunction with the degree of β-amylolysis have been used for the calculation of the exterior and interior chain lengths of glycogens. The interaction of glycogen with iodine and concanavalin-A and the enzymic degradation with salivary α-amylase have also been studied to examine possible quantitative relationships with degree of branching. A study of these properties would then provide additional methods for glycogen analysis. Amylose and amylopectin samples have been included for comparative purposes.

Part 1. Preparation of Glycogen Samples

The horse, ox and pig glycogens were supplied by Dr. R.A. Lawrie. These were extracted with hot 30% sodium hydroxide solution and purified by the method of Somogyi (122). The oyster glycogen was a commercial sample purchased from Nutritional Biochemical Corporation.

Dr. I.D.E. Storey kindly provided ram liver, capon liver and fowl liver extracts from which glycogen was obtained. Dr. P.W. Kent provided various human liver samples. Other samples of glycogen were supplied by Dr. D.J. Manners. The preparation of the amylose and amylopectin samples will be described in Section 5.

Extraction and Purification

The tissues were ground with sand, or macerated, and extracted three times with boiling water (1 g. tissue to 5 ml. water) in an atmosphere of nitrogen. The aqueous extracts obtained by filtration through muslin and then kieselguhr were combined and the glycogen precipitated by the addition of ethanol (3 vol.). After centrifugation, the supernatant liquid was discarded and the glycogen was deproteinised by treatment with 4% trichloroacetic acid at 0° for ca. 20 hrs. The precipitated and coagulated protein was removed by centrifugation, and the glycogen then precipitated by the addition of ethanol (3 vol.). It was purified by six further precipitations with ethanol and dried with ether.

Purity of Glycogens

All the glycogens used in this work were 96-100% pure by acid hydrolysis with the exception of two samples, Arenicola marina (88%) and a hot water extracted normal human liver glycogen (92%). Glucose was the only sugar detected in acid hydrolysates, examined by descending chromatography using solvent 1. with spray 1. In the case of the two

glycogens of low glucose content it was assumed that these glycogens must have high ash contents. None of the glycogen samples had any detectable reducing power.

Part 2. End-group Assay of Glycogen-type Polysaccharides by Periodate Oxidation

Introduction

End-group assay of branched polysaccharides of the glycogen type by periodate oxidation was developed by Halsall, Hirst and Jones (123). The basis of the estimation is that on oxidation each non-reducing end-group which has free hydroxyl groups at positions 2, 3 and 4 reduces 2 moles of periodate with the liberation of 1 mole of formic acid. Estimation of the formic acid therefore gives a direct measure of the number of end-groups in the molecule. The single reducing group gives rise to two moles of formic acid but this can be neglected owing to the size and degree of branching of the molecule.

Early workers found that use of sodium metaperiodate often resulted in a periodate reduction and formic acid release greater than expected. This effect, termed overoxidation, was avoided by Hirst and coworkers (124) by the use of the sparingly soluble potassium metaperiodate. Oxidation of glycogen by this method was found by Bell and Manners (125) to be complete within 250-300 hr. and the method is established as one which gives a minimum of overoxidation.

To limit overoxidation during oxidation with sodium metaperiodate there are several necessary conditions. Oxidations should be carried out (i) in the dark, (126) (ii) with a low concentration of periodate, (127) (iii) at a low temperature (127) and (iv) with a final formic acid concentration of less than 0.002 N to avoid its oxidative breakdown (128).

Several methods of sodium periodate oxidation have been used by different workers, this type of oxidation having two advantages over the potassium method in that the periodate reduction can also be measured, and the rate of oxidation is usually faster.

In the present study, the chain lengths of several glycogens have been measured by potassium and sodium periodate oxidation, and different conditions of sodium periodate oxidation have been critically compared.

Experimental

The following methods were used:

1. Oxidation with potassium metaperiodate

Glycogen (ca. 250 mg.) was oxidised in brown bottles with a mixture of 20 ml. of 8% sodium metaperiodate and 80 ml. of 5% potassium chloride solution at room temperature, with gentle agitation, as described by Bell and Manners (125). Aliquots (15 ml.) were removed for estimation of formic acid after approximately 150, 200, 250, 300 and 350 hr. Ethylene glycol (1.5 ml.) was added for destruction of excess periodate,

before titration.

2. Oxidation with sodium metaperiodate

(a) Method: Manners and Archibald (129).

Glycogen (ca. 100 mg.) dissolved in water (22 ml.) was oxidised with 3 ml. of 8% sodium metaperiodate in the dark at 2°. Samples (4 ml.) were removed for estimation of formic acid, ethylene glycol (1 ml.) being added to reduce excess periodate.

(b) The above method was also carried out at room temperature.

(c) Method: Polglase, Smith and Tyler (130).

Glycogen (ca.250 mg.) was dissolved in 8 ml. water and cooled to 2°. This solution was oxidised in the dark with 17 ml. of 5% sodium metaperiodate. Samples (3 ml.) were removed for the estimation of formic acid after addition of ethylene glycol (0.5 ml.).

(d) Method: Perlin (131).

Glycogen solution (25 ml.; ca. 4 mg./ml.) was oxidised with 20 ml. of 5% sodium metaperiodate at 20° in the dark. Samples (10 ml.) were analysed for formic acid after the addition of ethylene glycol (1 ml.).

(e) Method: Abdel-Akher and Smith (127).

Glycogen (250 mg.) was oxidised at 2° with 55 ml. of 5% sodium metaperiodate in a total volume of 250 ml. Samples (20 ml.) were analysed for formic acid after the addition of 0.5 ml. ethylene glycol.

(f) Method: Potter and Hassid (132).

Amylopectin (ca. 400 mg.) was dissolved in 3% sodium chloride (10 ml.) and oxidised in the dark with 10 ml. of 8% sodium metaperiodate at 2°. Aliquots were removed for the estimation of periodate reduction and formic acid release.

Reagent controls, without polysaccharide, were set up for each of the above methods, and recorded titres are corrected for the corresponding blank values.

Polysaccharide solutions, when necessary, were adjusted to pH 5.8 with 0.01 N sodium hydroxide or 0.01 N formic acid before oxidation. After the addition of ethylene glycol, solutions were left in the dark for 0.5 hr. before titration.

Results

1. Oxidations with potassium metaperiodate

The results of a number of oxidations by Method 1 are given in the following Table. Sodium hydroxide solution - 0.01 N was used in the titrations.

Table III. 1

Glycogen sample	Time (hrs.)	Formic Acid (%)	$\overline{CL}$ (glucose residues)
Oyster (257.5 mg.)	164	2.38	11.9
	212	2.53	11.2
	312	2.70	10.5
	408	2.72	10.4
Oyster (75.0 mg.)	312	2.52	11.3
	408	2.58	11.0
Horse l.dorsi muscle (251.2 mg.)	240	1.68	16.9
	312	1.71	16.6
	408	1.71	16.6
Rabbit liver VII (322.8 mg.)	216	2.0	14.2
	264	2.04	13.9
	312	2.04	13.9
Rabbit liver VIII (319.7 mg.)	216	2.14	13.3
	264	2.18	13.0
	312	2.22	12.8
Rabbit liver IX (327.3 mg.)	264	2.17	13.1
	312	2.18	13.0
Rabbit muscle III (306.2 mg.)	216	2.14	13.3
	264	2.18	13.0
	312	2.18	13.0
KP Amylopectin* (252.4 mg.)	216	1.21	23.4
	264	1.23	23.1

* From potato starch (var. Kerr's pink)

Horse and ox muscle glycogens (ca. 75 mg.) were oxidised

with a suspension of potassium metaperiodate (5 ml. of sodium metaperiodate and 20 ml. of potassium chloride) under the above conditions. The horse l.dorsi (post-rigor)glycogen was used as a control giving the following results:

	Time (hr.)	Formic Acid (%)	$\overline{CL}$
	216	1.68	16.9
	240	1.71	16.6
Wt. of sample - 251.9 mg.	264	1.71	16.6

Table III. 2

Horse muscle glycogens

	Wt. (mg.)	Time (days)	Formic Acid (%)	$\overline{CL}$
pre-rigor	77.2	10	1.69	16.8
Horse l. dorsi				
post-rigor		control		16.6
pre-rigor	74.4	10	1.69	16.8
Horse diaphragm				
post-rigor	76.9	10	1.71	16.6
pre-rigor	101.2	13	1.72	16.5
Horse psoas				
post-rigor	76.9	10	1.66	17.1
Horse heart, post-rigor	73.7	10	1.72	16.5
pre-rigor	78.2	10	1.72	16.5
Ox psoas				
post-rigor	59.8	13	1.93	14.7

Table III. 2 continued

Horse muscle glycogens

		Wt.(mg.)	Time(days)	Formic Acid (%)	$\overline{CL}$
Ox sternocephalicus	pre-rigor	44.5	10	1.53	18.6
	post-rigor	55.7	10	1.89	15.0

After a further 48 hr. the formic acid concentration was measured again; the results were the same as in Table III. 2; the oxidation was therefore complete.

The results for several other glycogen samples are given below:

Table III. 3

Source	Time of oxidation (days)	Formic acid (%)	$\overline{CL}$
Pig liver (sugar fed)	17	1.83	15.5
Pig liver (normal)	13	1.81	15.7
	17	1.87	15.2
Pig (dorsi muscle)	13	1.81	15.7
	17	1.83	15.5
Rabbit liver XIV	13	1.64	17.3
Rabbit liver XV	13	1.86	15.3

2. Oxidation with sodium metaperiodate

Oxidation of oyster, skate and horse l.dorsi glycogen by Method 2a.

Table III. 4

Source	Time (days)	Formic Acid (%)	$\overline{CL}$
Oyster (102.7 mg.)	9	2.56	11.1
	13	2.61	10.9
	15	2.73	10.4
	17	2.73	10.4
Skate liver (102.4 mg.)	9	2.06	13.8
	13	2.10	13.5
	17	2.10	13.5
Horse l.dorsi muscle (post-rigor) (105.1 mg.)	7	1.55	18.3
	10	1.63	17.4
	14	1.64	17.3
	20	1.69	16.8

Oxidation of oyster and rabbit liver VII glycogens by Methods 2a,b, c,d and e.

Table III. 5

Oyster glycogen ($\overline{CL}$ 10.4)*

Method	Wt. of glycogen (mg.)	Time (hr.):	49	96	168	216	216	260	336	696
					Apparent chain length					
a	99.6		14.9	12.8	11.9	-	11.3	10.5	-	10
b	100.0		12.3	10.8	10.2	-	9.9	9.4	-	-
c	264.3		10.9	10.1	9.6	-	8.9	8.7	-	6.8
d	103.3		10.4	9.8	8.8	-	8.3	7.6	-	5.0
e	264.2		-	-	-	10.1	-	9.3	8.6	7.8

Rabbit liver VII glycogen ($\overline{CL}$ 13.9)*

a	102.0		21.6	18.2	16.9	-	16.4	16.0	-	14.0
b	100.2		18.4	16.7	15.6	-	15.0	14.7	-	12.5
c	258.6		16.3	14.7	13.3	-	12.1	11.4	-	8.10
d	101.1		14.7	13.2	12.1	-	11.3	10.6	-	6.10
e	261.9		-	-	-	12.9	-	11.6	10.3	8.5

* By potassium periodate oxidation

Oxidation of oyster, rabbit liver VII and Ascaris lumbricoides glycogens by Method 2e.

The scale of the oxidation mixtures was reduced for 100 mg. polysaccharide.

Table III. 6

Sample	Time (days)	Formic acid (%)	$\overline{CL}$	$\overline{CL}$ (KIO_4)
Oyster	9	2.73	10.4	
(105.2 mg.)	12	2.81	10.1	10.4
	15	3.11	9.1	
Rabbit liver VII	9	2.04	13.9	
(105.4 mg.)	12	2.31	12.3	13.9
	15	2.90	9.8	
Ascaris lumbricoides	9	2.10	13.5	
(104.0 mg.)	12	2.63	10.8	12.0
	15	3.02	9.4	

Oxidation of AK human liver glycogen (see Section 4) by Method 2d.

Oxidation mixtures were set up as follows:

(i) 102.8 mg. was oxidised under standard conditions.
(ii) 102.8 mg. was oxidised with twice the amount of sodium metaperiodate in a total volume of 100 ml. (i.e. at half polysaccharide concentration).
(iii) 102.8 mg. was oxidised as (i) but at 37°.

A graph of formic acid production against time was prepared, and by extrapolation to zero time, a $\overline{CL}$ value was obtained.

Table III. 7

	Time (hr.)	Formic acid (%)	Apparent $\overline{CL}$	Extrapolated $\overline{CL}$
(i)	49	2.20	12.9	
	95	2.37	12.0	13.9
	140	2.58	11.0	
	180	2.65	10.7	
(ii)	49	2.18	13.0	
	95	2.41	11.8	13.9

Table III. 7 continued

Time (hr.)	Formic acid (%)	Apparent $\overline{CL}$	Extrapolated $\overline{CL}$
140	2.59	11.0	
180	2.67	10.6	
(iii) 24	2.55	11.1	
49	3.38	8.4	13.8
75	5.16	5.5	
95	6.97	4.1	

Oxidation of Trehalose by Method 2d

Since formic acid was being released from other residues, the oxidation of trehalose as a model-compound was examined. It was oxidised under the conditions of Method 2d, the results being expressed as apparent moles of formic acid liberated per mole of the disaccharide. The theoretical formic acid liberation was obtained by extrapolation of the apparent value to zero time. The results are given in Table III. 8.

Weight of trehalose = (a) 23.2 mg.
(b) 11.6 mg.
(c) 8.0 mg.

Table III. 8

Time (hr.)	Apparent moles formic acid (a)	(b)
25.8	2.11	-
48.5	2.14	2.19
74.3	2.16	2.24
94.3	2.19	2.29
116.8	2.18	2.27

Table III. 8 continued

Time (hr.)	Apparent moles formic acid (c)
4	2.26
19.7	2.29
23	2.29
29	2.28

Oxidation of Glycogen and Amylopectin by Method 2d

The polysaccharides were oxidised under normal conditions at 20° giving the following results:

Table III. 9

Glycogens	Time(hr.)	Formic acid(%)	Apparent CL	Extrapolated CL
SK Human liver (70 mg.)	50	4.66	6.1	
	97	4.79	5.94	6.3
	146	4.79	5.94	
	191	5.05	5.62	
Ram liver (97.6 mg.)	50	2.16	13.2	
	97	2.35	12.1	14.3
	146	2.57	11.1	
	191	2.92	9.7	
Rabbit liver VIII (93.6 mg.)	50	2.47	11.5	
	97	3.02	9.4	13.5
	146	3.69	7.7	
	191	4.74	6.0	
Cardium tuberculatum (Muscle du pied) (93.0 mg.)	48	2.45	11.6	
	92	2.66	10.7	12.4
	122	2.74	10.4	
	147	2.85	10.0	
Cardium tuberculatum (Muscle adducteur) (85.5 mg.)	48	2.88	9.9	
	92	3.34	8.5	11.3
	122	3.71	7.7	
	147	3.90	7.3	

Table III. 9 continued

Amylopectin	Time(hr.)	Formic acid(%)	Apparent $\overline{CL}$	Extrapolated $\overline{CL}$
Potato (KP) (242 mg.)	48	1.41	20.2	
	74	1.60	17.8	24.4
	95	1.91	14.9	
	116	2.06	13.8	
Maize (187 mg.)	48	2.06	13.8	
	74	2.37	12.0	18.8
	95	2.82	10.1	
	116	3.44	8.3	
KP amylopectin β-amylase limit-dextrin (96.6 mg.)	48	3.41	8.3	
	73	3.64	7.8	9.5
	94	4.15	6.8	
	116	4.30	6.6	

Oxidation of KP Amylopectin and Waxy Maize Starch IV by Method f

Periodate reduction was estimated as described in Section 2.

Table III. 10

KP Amylopectin (382 mg.)

Time (hr.)	Periodate reduction (moles)	Formic acid (%)	$\overline{CL}$
6	0.90	0.93	30.5
25	0.98	1.11	25.6
34	1.04	1.25	22.8
Waxy Maize Starch IV (387 mg.)			
6	1.01	0.94	30.2
25	1.09	1.08	26.3
34	1.09	1.18	24.1

Discussion

In the above experiments the end-point of the formic acid - sodium hydroxide titration was taken to pH 5.8 as this lay on the vertical portion of the acid-alkali titration graph. Anderson, Greenwood and Hirst (133) suggested that pH 6.25 should be regarded as the correct end-point but the corrected titre difference required to change the pH from 5.8 to 6.25 was found to be of the order of 0.01-0.03 ml. in a titre of 1.5 ml. 0.01 N alkali, which is negligible. Polysaccharide solutions were therefore adjusted to pH 5.8 on a pH meter before oxidation.

Oxidation of glycogen with potassium periodate was found to be complete in 250-300 hr. (Table III.1) and there was no significant overoxidation observed even after 400 hr. There was some slight lack of reproducibility in reducing the oxidation scale by one quarter, a chain length value of 11.0 being obtained in contrast to 10.4 on the normal scale. Smaller titres were obtained on the small scale so that a slightly reduced accuracy is to be expected.

Small scale oxidation (ca. 75 mg.) of the horse muscle glycogens was necessary because of the small amounts of material available. To ensure maximum possible accuracy large aliquots (10 ml.) were removed after 10 and 12 days giving titres greater than 1.0 ml. alkali. Support for the accuracy of the above method is the value (16.6) obtained for the post-rigor sample of horse l. dorsi glycogen, which is in excellent agreement with those from normal scale potassium and

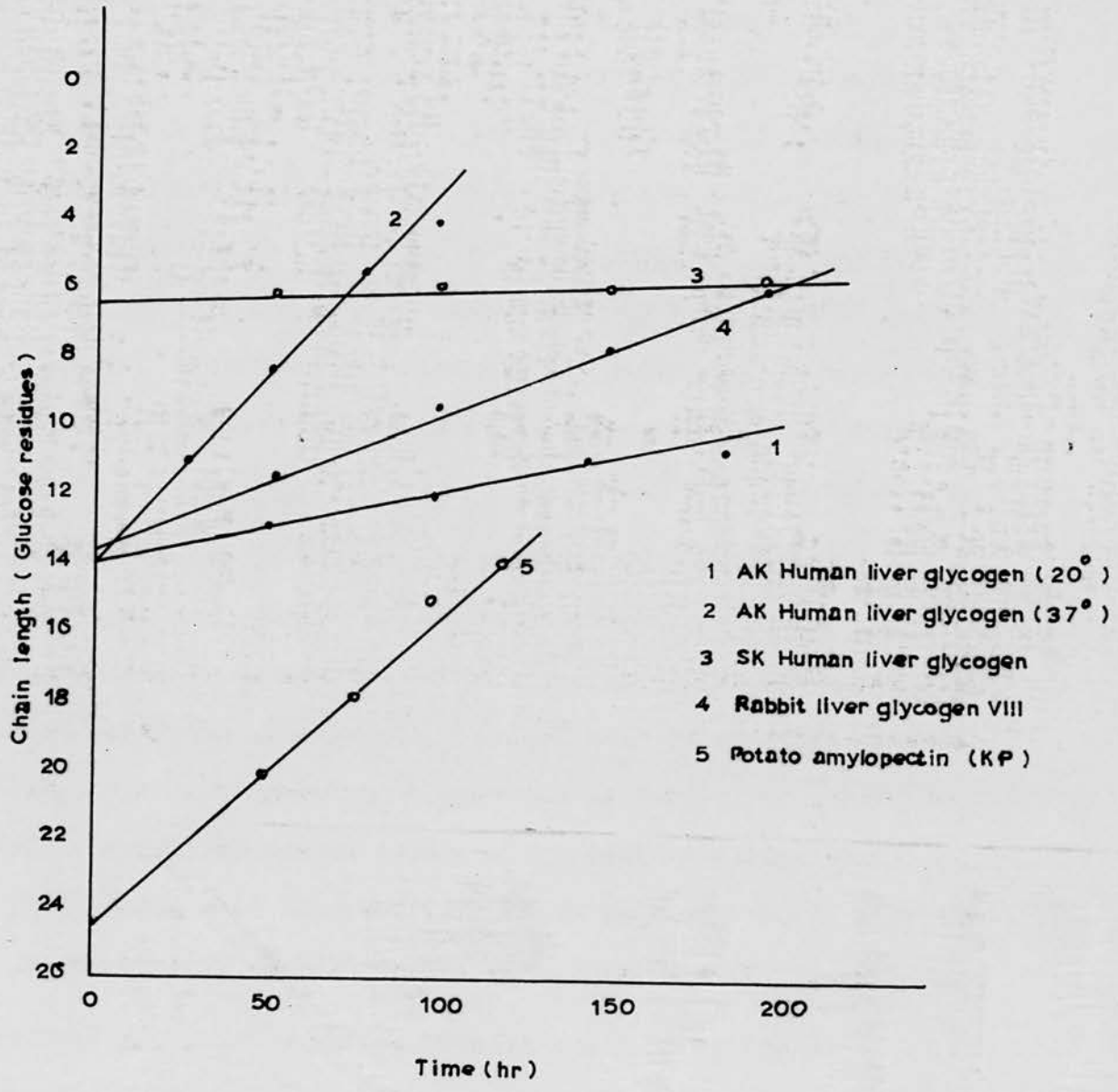
sodium periodate oxidations (16.6 and 16.8 respectively).

The chain lengths in Table III.1, apart from the horse muscle glycogens, are in the range 12 ± 2 glucose residues, in agreement with the majority of previously reported results. The chain length values in Table III.3 are all greater than 15.0 and as these samples have β-amylolysis limits greater than 50% (see Part 4) this indicates slightly longer exterior chains. Samples of liver glycogen obtained from a pig fed on a special sugar diet, and from two rabbits (XIV and XV) which had been fasted and fed galactose by stomach-tube, also had chain lengths greater than 15 glucose units.

Oyster and rabbit liver VII glycogens were oxidised with sodium metaperiodate under five different conditions (Table III.5); the rates of oxidation were in the order: d>c>e>b>a. and significant overoxidation occurred in all the methods apart from Method (a). In the case of this latter method, oyster glycogen was fully oxidised after 260 hr.; oxidation of rabbit liver glycogen VII, on the other hand, was not quite complete even after 696 hr. This supports the previous conclusion of Manners and Archibald that oxidation proceeds at different rates with different glycogen samples. Method (b), in both cases, gave a steadily increasing production of formic acid, which was due to increased temperature.

The amount of periodate per mg. polysaccharide under the conditions used by Smith (e) and Perlin (d) is approximately three

FIGURE 7


Chain length (Glucose residues)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
50
100
150
200
Time (hr)
1
2
3
4
5
1 AK Human liver glycogen (20°)
2 AK Human liver glycogen (37°)
3 SK Human liver glycogen
4 Rabbit liver glycogen VIII
5 Potato amylopectin (KP)

times that in Method (c) and six times that in Methods (a) and (b). That Methods (d) and (c) are faster than Method (e) must be due in the former case to the higher temperature, and in the latter to the high polysaccharide concentration.

The results in Tables III.5 and III.6 indicate that formic acid production in Method (e) does not become constant and although the nine day values for oyster and rabbit liver VII glycogens (Table III.6) agree with the values obtained by the potassium method, that of the Ascaris lumbricoides glycogen is not in agreement with this value. The report of Abdel-Akher and Smith (127) that this method gave complete oxidation but not overoxidation after 180 hr. at 0° and that the formic acid production became constant is not confirmed in the present study.

The methods of Polglase and coworkers (c) and Perlin (d) gave approximately linear increases in formic acid production up to 250 hr. The former method gave no indication of the chain length, overoxidation being very rapid. Method (d), also gave rapid overoxidation, but values in reasonable agreement with those from potassium periodate oxidation could be obtained by extrapolation of the linear portion of the apparent chain length versus time graph (Fig. 7) to zero time. This method was therefore investigated further.

Oxidation of a human liver glycogen (A.K. see Section 4) by Method (d) at two different polysaccharide concentrations gave identical values for the average chain length. Even at 37°, when oxidation was very rapid a linear graph was obtained (Fig. 7) up to 75 hr., which

gave a value similar to those obtained at 20°.

The following Table compares the results obtained by the application of Method (d) to a number of polysaccharides with those from potassium periodate oxidation:

Table III. 11

Sample	$\overline{CL}$ by method (d)	$\overline{CL}$ by potassium periodate oxidation
A.K. Human liver glycogen	13.9	14.5
S.K. Human liver glycogen	6.3	6.3*
Rabbit liver VII glycogen	14.7	13.9
Rabbit liver VIII glycogen	13.5	12.8
Oyster glycogen	11.0	10.4
KP Amylopectin	24.4	23.1
KP Amylopectin β-dextrin	9.5	9.0**

* Result obtained by Dr. D.J. Manners

** Calculated from the β-amylolysis limit, and original chain length.

All the above results are believed to be accurate to within ± 1.0 glucose residue.

The above data compare favourably with those obtained by the potassium periodate method. Method (d) has the advantage that it is fairly rapid and gives an unambiguous result while most other sodium periodate methods tend to give a slow increase in formic acid production

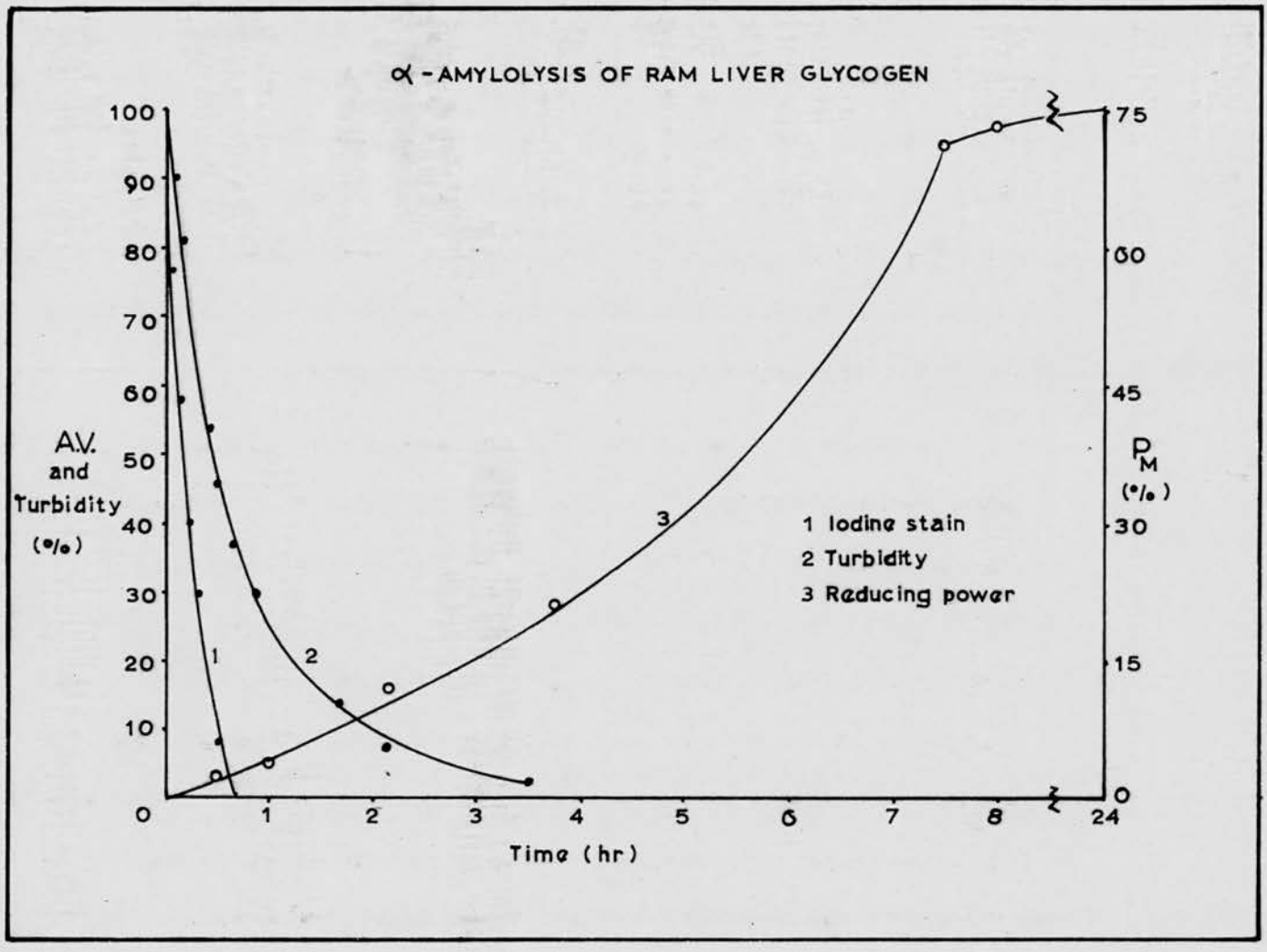
and since different samples are oxidised at different rates it is difficult to determine when oxidation is complete, unless periodate reduction is measured at the same time.

Variation in temperature does not seem to affect the results as long as a constant temperature is maintained throughout the oxidation. Temperatures of 15-20° are to be preferred to higher or lower temperatures the former causing a rapid decrease of apparent chain length with time and thus an increased error, and the latter making the oxidation too slow.

Oxidation of trehalose with Method (d) shows that 1.15 moles formic acid (theoretical 1.0) may be produced from each end-group. It is possible that similar overoxidation of all the dialdehyde residues in the glycogen molecules may take place, and account for the linear portion of the graph in Figure 7.

The results for the oxidation of two amylopectin samples by the method of Potter and Hassid are given in Table III.10. These workers reported that oxidation was complete after 25 hr. on the assumption that the rate of oxidation of maltose was the same as that of polysaccharides such as glycogen and amylopectin. The results obtained by the present author show that this is not the case, chain lengths of 25.6 and 26.3 being obtained for KP amylopectin and waxy maize starch IV respectively. By potassium periodate oxidation the respective values were 23.1 and 22.0.

FIGURE 8


α-AMYLOLYSIS OF RAM LIVER GLYCOGEN
A.V. and Turbidity (%)
P_M (%)
Time (hr)
1 Iodine stain
2 Turbidity
3 Reducing power
100
90
80
70
60
50
40
30
20
10
0
75
60
45
30
15
0
1
2
3
4
5
6
7
8
24

Part 3 Studies on Salivary α-Amylolysis

Introduction

α-Amylases catalyze the random hydrolysis of α-1,4-glucosans; this is characterized by a rapid decrease in turbidity, in viscosity and in iodine staining power accompanied by a slow increase in reducing power (for example, see Figure 8). Oligosaccharides of D.P. ca. 6-12 are formed in the initial (dextrinisation) stages and are broken down in a later (saccharification) stage to smaller branched dextrins (D.P. ca. 5) and sugars.

Salivary α-amylase in low concentration acting on glycogen or amylopectin liberates 6^3-α-maltosylmaltotriose as the smallest oligosaccharide (11). With a high enzyme concentration, the smallest oligosaccharide produced is 6^3-α-glucosylmaltotriose (64). Thus, associated with every α-1,6-linkage there are two or three α-1,4-linkages which are resistant to enzymic attack. The degree of α-amylolysis should therefore be inversely proportional to the percentage of resistant linkages and therefore also to the percentage of 1,6-linkages in the molecule (P_R value).

The salivary α-amylolysis of a number of glycogen samples has been carried out to investigate the relationship between the P_M and P_R values.

Experimental

1. Examination of the Enzyme (see Section 2).

Activity Determination (Method of Fischer and Stein (108)).

One unit of activity is defined as the quantity of enzyme which liberates 1 mg. of 'apparent' maltose from a 1% solution of soluble starch in 3 minutes at 35°.

The following digest was prepared: 1% AR soluble starch solution (25 ml.) and 0.5% sodium chloride (3 ml.) were preheated to 35° and incubated with α-amylase solution (2 ml.; 0.019 mg./ml.). Aliquots removed after 27 and 30 minutes gave reducing powers equivalent to the liberation of 12.8 mg. 'apparent' maltose in 30 minutes. This is equivalent to an activity of 34 units/mg. enzyme.

Maltase Activity

The enzyme was incubated at 35° with maltose in the following digest: Maltose (25.0 mg.), α-amylase (0.5 ml.; 5.5 mg./ml.), 0.5% sodium chloride solution and water to 50 ml. were incubated at 35°.

The hydrolysis of maltose was 2 and 3% respectively after 24 and 48 hr. Thus, under the above conditions, which were used in all digests, there was a trace of maltase activity.

2. α-Amylolysis of Glycogens

Chromatograms developed with spray 1. showed the presence of glucose, maltose, maltotriose and higher oligosaccharides.

Control digests were used in every case to standardize

the results and aliquots (5 ml.) were removed at approximately the same time intervals. Enzymic action was found to be complete after 9 hr. after which there was a slow increase in reducing power, probably due to the presence of maltase.

Results and Discussion

The 24 hr. P_M values obtained for a variety of glycogens together with two samples of amylose and two of amylopectin are summarized in the following Table.

Table III. 12

Glycogen samples	P_M value (%)	P_R value** (%)	P_M calculated	P_R calculated
SK liver*	33	15.9	34	16.4
Rabbit liver VII (48% β-limit dextrin)	38	15.2**	38	15.3
KP Amylopectin β-dextrin	56	10.5	60	11.6
Rabbit liver VII (37% β-dextrin)	57	11.6**	55	11.3
Rabbit liver VII (22% β-dextrin)	62	9.4**	65	10.3
Oyster	68	9.5	65	9.0
AK liver*	74	6.9	77	7.8
AK kidney*	76	7.0	77	7.4
Horse psoas (post-rigor)	76	5.9	82	7.4
Rabbit liver VII (7% β--dextrin)	76	7.9**	73	7.4
D_h (Section 4)	77	6.7	78	7.1
AK liver (duplicate)*	77	6.9	77	7.1
Horse l.dorsi (pre-rigor)	78	6.0	82	6.9

Table III. 12 continued

Glycogen samples	P_M value (%)	P_R value** (%)	P_M calculated	P_R calculated
Horse heart (post-rigor)	78	6.1	81	6.9
Horse psoas (pre-rigor)	79	6.2	81	6.7
Pig liver (normal)	79	6.6	79	6.7
Oxsternocephalicus (post-rigor)	79	6.7	78	6.7
Ox psoas (post-rigor)	79	6.8	78	6.7
Rabbit liver VII	79	7.3	76	6.7
Horse diaphragm (pre-rigor)	80	6.0	82	6.5
Horse diaphragm (post-rigor)	80	6.0	82	6.5
Human liver Dc*	80	6.9	77	6.5
Pig dorsi muscle	81	6.5	79	6.3
Human liver Nc*	81	6.6	79	6.3
Ox psoas (pre-rigor)	82	6.1	81	6.1
Horse l.dorsi (post-rigor)	83	6.0	82	5.9
Ox sternocephalicus (pre-rigor)	83	5.4	85	5.9
Pig liver (sugar fed)	84	6.5	79	5.7
KP Amylopectin (Duplicate)	89	4.2	90	4.6
KP Amylopectin (Duplicate)	93	4.2	90	3.8
Maize amylopectin	88	5.3	85	4.8
KP Amylose	112	0	111	-
Maize Amylose	114	0	111	-

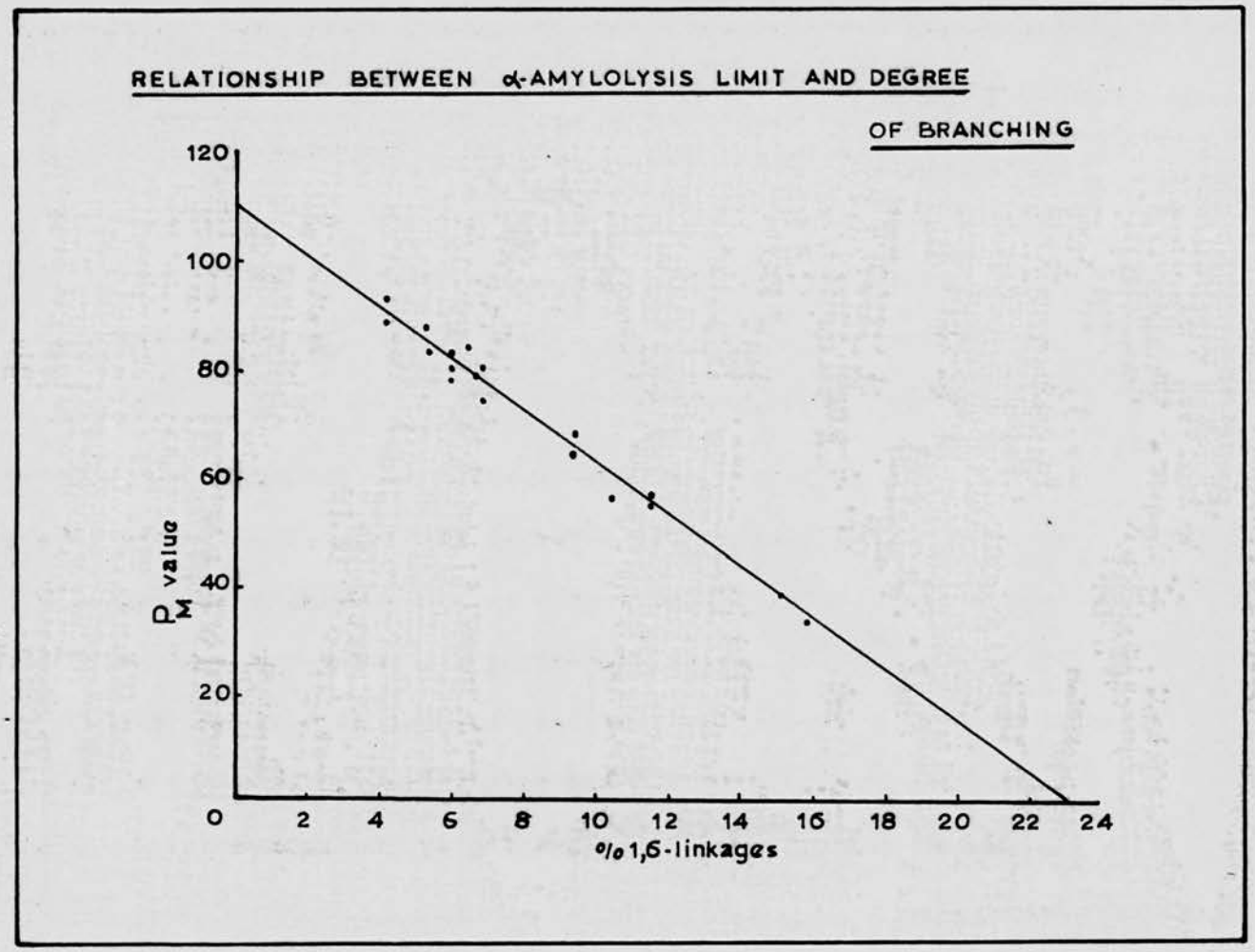
* Human glycogen, see Section 4.

* Values calculated from the degree of β-amylolysis.

** P_R values are calculated from the relationship.

$$\% \text{ 1,6-linkages } (P_R) = \frac{100}{CL}$$

FIGURE 9


RELATIONSHIP BETWEEN α-AMYLOLYSIS LIMIT AND DEGREE OF BRANCHING
120
100
80
60
40
20
0
P_M value
2
4
6
8
10
12
14
16
18
20
22
24
% 1,6-linkages

Graphical representation of these results shows that there is a significant relationship between the measured P_M value and the P_R value calculated from periodate oxidation results (Figure 9). The values for amylose, which are greater than 100% are due to the production of a significant amount of glucose which has a reducing power twice that of maltose. The high P_M values for amylopectin are in good agreement with the low P_R value and the low P_M value for the 48% β-amylase limit dextrin of rabbit liver VII glycogen illustrates the effect of an increased percentage of resistant linkages.

The highest P_M values observed for glycogen were 83% for ox sternocephalicus muscle glycogen and 84% for pig liver glycogen (sugar fed). The former sample has an extremely low degree of branching ($\overline{CL}$ 17) almost resembling that of maize amylopectin ($\overline{CL}$ 19). Experimental error cannot account for the significant differences in their P_M values, 83 and 88% respectively, which may therefore be due to differences in positioning of the branch points in the molecule.

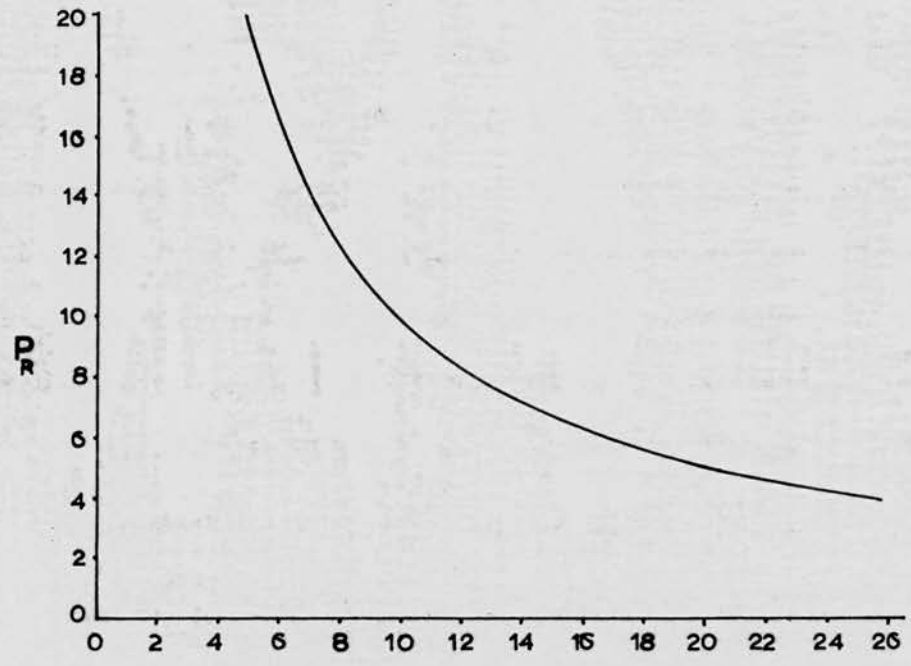
Statistical analysis, by the method of least squares, of the data in columns 1 and 2 in Table III.12 gives the following equations for the P_M versus P_R graph:

$$P_M = 110.5 - 4.8\,(P_R) \quad \ldots\ldots\ldots \quad 1.$$

$$P_R = 23.3 - 0.21\,(P_M) \quad \ldots\ldots\ldots \quad 2.$$

In equation 1. when the P_R value is zero, the corresponding

FIGURE 10


20
18
16
14
12
10
8
6
4
2
0
D_R
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Average chain length (glucose residues)

P_M value is 110.5; this is in good agreement with the observed P_M values for amylose, which were not used in derivation of the formula. The P_R value from equation 2. when the P_M value is zero (i.e. complete resistance to α-amylolysis) is 23.3 equivalent to a chain length of 4.3 glucose residues. This is very significnnt since the smallest resistant molecule, containing a 1,6-linkage, produced during α-amylolysis is the tetrasaccharide 6^3- α - glucosylmaltotriose.

Calculated values of P_M and P_R are shown in columns 3 and 4 of Table III.12. The standard deviation (σ) for equation 1. is 2.60 and for equation 2. is 0.58. Statistically, 95% of the results are expected to be within σ x 1.645, and any values outside this range represent significant differences. This factor is referred to as the maximum possible error (M.P.E.).

M.P.E. (equation 1.) = $\pm$ 4.3 in P_M value.

M.P.E. (equation 2.) = $\pm$ 1.0 in P_R value.

The P_R value determined by α-amylolysis will therefore be correct to within $\pm$1 under the present conditions. Since the P_R value is indirectly proportional to the chain length, this latter value increases with decrease of P_R value (Figure 10).

Below a P_R value of 7% the error in chain length becomes greater than $\pm$2 glucose residues, but as the P_R value increases, this error decreases rapidly to $\pm$1 glucose residue with 10% and $\pm$0.6 glucose residues with 12% of 1,6-linkages. For glycogens with

chain length ≤ 14, therefore, the method is useful for the rapid determination of chain length values on small amounts of material.

The low P_R values of amylopectins makes determination of chain lengths by direct α-amylolysis impossible. A modified method has therefore been developed which gives greater accuracy.

Polysaccharide (ca. 50-90 mg.) was incubated with barley β-amylase (25 units/mg.) at pH 4.6 in a total volume of 30 ml. After incubation for 24 hr. the enzyme was denatured by heating; the maltose production was then estimated. A 20 ml. aliquot was removed, adjusted to pH 7.0, and reincubated at 37° with salivary α-amylase (3 units/mg. polysaccharide) in 0.5% sodium chloride (5 ml.) in a total volume of 50 ml. The enzyme action was complete after 9 hr. when the total reducing sugar was estimated as maltose. The increase in maltose content gave the P_M value of the β-limit dextrin from which the average chain length was calculated using formula 2. The chain length of the polysaccharide was then calculated from this value and the β-amylolysis limit;

$$\overline{CL} = \frac{\overline{CL}\ \text{dextrin}}{(100 - \beta\text{-limit}\ (\%))} \times 100$$

The values obtained for two amylopectins and five glycogens are given in Table III. 13.

Table III. 13

	Wt. polysaccharide (mg.)	β-limit (%)	P_M value (%)	P_R value (%)	$\overline{CL}$ (dextrin)	$\overline{CL}$	$\overline{CL}$ by periodate oxidation
Glycogens							
Oyster	63.1	39	37.7	15.4	6.5	10.7	10.5
Mytilus edulis	60.2	53	44	14.1	7.1	15.1	-
Rabbit liver VII	60.3	51	42	14.5	6.9	14.1	13.9
Rabbit muscle III	69.7	49	37.6	15.4	6.5	12.7	13.0
Ram liver	61.6	46	40.4	14.8	6.8	12.6	14.3
Amylopectins							
KP Amylopectin	81.5	60	63	10.1	9.9	24.8	23-24
Maize	46.6	57	60	10.7	9.3	21.6	19.0

The results in Table III.13 are in good agreement with those obtained by periodate oxidation, the greatest errors being with the ram liver glycogen and maize amylopectin samples. In the case of the latter, the error could be partly due to the relatively small quantity of polysaccharide (20 mg.) in the α-amylolysis digest. Experimental error at this concentration could have a significant effect on the P_M value.

The similarity in the dextrin chain lengths of the glycogen

samples is striking since they agree within 0.5 glucose residues. It may be that many glycogens are similar in this respect, and differ mainly with regard to exterior chain length. The latter would depend upon the nutritional state of the animal at the time of death; the unusually long exterior chain length of liver glycogen from mammals fed on rich carbohydrate diets has already been noted.

Within certain limitations, the above investigations show that α-amylolysis may provide a useful indication of the degree of branching in a glycogen-type polysaccharide.

Part 4 β-Amylolysis of Glycogen and Amylopectin

Introduction

The occurrence and mode of action of β-amylase has been described in Section 1. The β-amylolysis limit has been used in the present study, in conjunction with the average chain length, to calculate the lengths of exterior ($\overline{ECL}$) and interior chains ($\overline{ICL}$) of glycogens and amylopectins, thus obtaining more detail of molecular fine structure.

Results

The results of the β-amylolysis of glycogens and amylopectins from various sources, as described in Section 2, are given in Table III.14. Chain lengths were obtained from sodium or potassium periodate oxidation results.

Table III. 14

Glycogen	β-Amylolysis limit (%) 24 hr.	48 hr.	$\overline{CL}^a$	$\overline{ECL}^b$	$\overline{ICL}^c$
1. Invertebrate sources					
Arenicola marina	36	36			
Oyster	39	40	10-11	7	2-3
Cardium tuberculatum:					
muscle du pied	44	45	12	8	3
muscle adducteur	46	46	11	7-8	2-3
Trichomonas gallinae	-	47	13*	8-9	3-4
Tetrahymena pyriformis II	-	48	13*	9	3
Mytilus edulis XI	47	48			
2. Liver glycogens					
Cod liver	44	44			
Ram liver	44	44	14	9	4
Skate liver	43	44	13-14	8-9	4
Rabbit liver XI	46	48	16*	10	5
Rabbit liver IV (alkali treated)	47	48	13*	9	3
Rabbit liver VIII	47	48	13	9	3
Pig liver I	48	48			
Rabbit liver X	48	49	12*	8-9	2-3
Rabbit liver IX	48	49	13	9	3
Rabbit liver VII	-	51	14	9-10	3-4
Pig liver (normal)	50	51	15	10	4
Rabbit liver XIV	50	52	17	11-12	4-5
Cat liver VI	52	52	12*	8-9	2-3
Rabbit liver XV	53	55	15	11	3
Pig liver (sugar fed)	56	56	15-16	11	3-4
3. Muscle glycogens					
Ox sternocephalicus (post-rigor)	43	44	15	9	5

Table III. 14 continued

Glycogen	β-Amylolysis limit (%) 24 hr.	48 hr.	$\overline{CL}$[a]	$\overline{ECL}$[b]	$\overline{ICL}$[c]
Rabbit muscle III	46	46	13	8-9	3-4
Horse psoas (post-rigor)	46	46	17	10	6
Horse l.dorsi (post-rigor)	45	47	17	10	5-6
Horse psoas (pre-rigor)	47	48	16-17	10-11	5
Horse heart (post-rigor)	45	48	16-17	10-11	5
Ox psoas (pre-rigor)	48	50	16-17	11	4-5
Ox psoas (post-rigor)	47	50	15	10	4
Pig dorsi muscle	50	50	15-16	10	4-5
Horse diaphragm (post-rigor)	49	51	17	11	5
Ox sternocephalicus (pre-rigor)	49	51	19	12	6
Horse diaphragm (pre-rigor)	50	53	17	11-12	4-5
Horse l.dorsi (pre-rigor)	52	53	17	11-12	4-5
Amylopectins					
Waxy sorghum I	-	56	25*	16-17	7-8
Waxy maize IV	57	57	22	15	6
Maize	-	57	19	13	5
Waxy maize I	58	58	22*	15	6
Waxy sorghum II	-	58	25	17	7
Waxy maize II	-	59	21	15	5
Potato (Kerrs Pink)	60	61	23	16-17	5-6

* Results of Manners and coworkers

[a]Average chain length in glucose units. [b]Average exterior chain length equal to the number of glucose residues removed by β-amylase + 2.5. [c]Average interior chain length equal to CL - exterior chain length - 1.

Discussion

The β-amylolysis limit alone gives little indication of fine structure, but together with the average chain length, it can

give useful information about the arrangement of the constituent chains in the molecule. The results in Table III.14 show that, in general, both the average exterior and interior chain lengths vary with source, increasing in the order, invertebrate glycogen, liver glycogen, muscle glycogen, amylopectin.

Three of the liver glycogen samples, rabbit livers XIV and XV and pig liver (sugar fed) have longer exterior chains than the others; the rabbits had been fed on galactose and the pig had a high sugar diet. These facts could account for the high values as it has been shown that the peripheral glucose residues are the most metabolically active (134). The above results suggest that high sugar diets may upset the balance slightly, resulting in an increase in the number of glucose units in the exterior chains.

The maize amylopectin has branching characteristics similar to those of several of the muscle glycogens. These latter polysaccharides however do not give characteristic amylopectin-type iodine stains (see Part 5), the iodine absorption being about one third that of an amylopectin. Other differences in structure must therefore exist.

Part 5 The Absorption Spectra of the Iodine Complexes of Glycogen and Amylopectin.

Introduction

In aqueous solution, glycogen gives a brown coloured complex with iodine the shade of which varies with biological source, from

a light yellow-brown with invertebrate glycogens to a dark red-brown with mammalian muscle glycogens. Amylopectins are much more iodophilic and tend to give a uniform reddish-violet iodine complex which varies only slightly in intensity from one sample to another.

The absorption spectra of the iodine complexes of several glycogens and amylopectins have been measured in an attempt to correlate these differences with structural features of the polysaccharides.

Results

All measurements were made under the standard conditions described in Section 2, in both aqueous solution, and in half-saturated ammonium sulphate solution.

Table III. 15

Iodine staining properties of polysaccharides in aqueous solution

Source	λ max(H_2O) (mμ)	E max(H_2O)	$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$
Invertebrate glycogens					
Arenicola marina	430-435	0.15	-	-	-
Mytilus edulis XI	435	0.16	-	-	-
Cardium tuberculatum:					
Muscle du pied	435-440	0.20	12	8	3
Muscle adducteur	440-445	0.18	11	7-8	2-3
Oyster	440	0.13	10-11	7	2-3

Table III. 15 continued

Source	λmax(H_2O) (mμ)	E max(H_2O)	$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$
Liver glycogens					
Pig liver I	435	0.24	-	-	-
Ram liver	435-440	0.42	14	9	4
Cod liver	445	0.18	-	-	-
Skate liver	445-450	0.14	13-14	8-9	4
Rabbit liver VIII	455-460	0.24	13	9	3
Rabbit liver XII	455-465	0.22	17*	10	6
Cat liver VI	465	0.34	12*	8-9	2-3
Rabbit liver X	475-480	0.28	12*	8-9	2-3
Rabbit liver IX	485	0.35	13	9	3
Muscle glycogens					
Rabbit muscle II	455-465	0.23	11*	7	3
Horse heart (post-rigor)	470	0.29	16-17	10-11	5
Horse diaphragm (pre-rigor)	475-480	0.32	17	11-12	4-5
Ox sternocephalicus (pre-rigor)	475-480	0.27	19	12	6
Horse diaphragm (post-rigor)	480-485	0.32	17	11	5
Horse l.dorsi (pre-rigor)	480-485	0.34	17	11-12	4-5
Ox sternocephalicus (post-rigor)	485	0.21	15	9	5
Ox psoas (pre-rigor)	490	0.34	16-17	11	4-5
Horse l.dorsi (post-rigor)	490	0.36	17	10	5-6
Rabbit muscle III	490-495	0.34	13	8-9	3-4
Horse psoas (post-rigor)	490-495	0.35	17	10	6

* Results of Manners and coworkers.

Table III. 16

Iodine staining of polysaccharides in presence of half-saturated ammonium sulphate

Source	Water		Ammonium sulphate				
	λ max	E max	λ max	E max	CL	ECL	ICL
Glycogens							
Rabbit liver VII	445-450	0.30	490	0.58	14	9-10	3-4
Rabbit liver XIV	460-465	0.35	500	0.48	17	11-12	4-5
Rabbit liver XV	470-485	0.51	505	0.75	15	11	3
Pig liver (sugar fed)	470-480	0.39	505-510	0.65	15-16	11	3-4
Pig liver (normal)	475-485	0.37	500	0.61	15	10	4
Ox psoas (post-rigor)	475-485	0.30	500-505	0.66	15	10	4
Horse psoas (pre-rigor)	490-495	0.32	490-500	0.56	16-17	10-11	5
Pig, dorsi muscle	490-500	0.45	495-505	0.75	15-16	10	4-5
Amylopectins							
Waxy maize II	525-530	0.85	550-555	1.07	21	15	5
Waxy maize I	530-535	0.95	540	0.97	22	15	6
Waxy maize IV	530-535	0.88	560	0.97	22	15	6
Waxy sorghum I	530-535	0.67	535-540	0.52†	25	16-17	7-8
Waxy sorghum II	530-535	0.96	560-565	1.18	25	17	7
Potato (KP)	535-540	1.13	535-545	0.65†	23	16-17	5-6
Potato (Epicure)	550	1.30	545	0.7†	24*	16	7

* Results of Banks and Greenwood

† Measured at half polysaccharide concentration

Table III. 17

Iodine staining properties of glycogen β-limit dextrins

Sample	Water		Ammonium sulphate	
	λ max. (mμ)	E max.	λ max. (mμ)	E max.
Foetal sheep liver glycogen	445-450	0.29	480-490	0.56
β-dextrin	425	0.11	415	0.37
Rabbit liver glycogen II	455	0.30	485	0.55
β-dextrin	420	0.21	425	0.50
Mytilus edulis (VI)	420-430	0.11	430	0.58
β-dextrin	420	0.03	420	0.29
Waxy maize starch (I)	530-540	0.92	545-555	0.60
β-dextrin	535-540	0.91	535	0.53

Effect of β-amylolysis on the iodine staining power

Digests containing rabbit liver VII glycogen and potato amylopectin (ca. 50 mg.) were incubated at pH 4.6 and 35° with barley β-amylase (2500 units) in a total volume of 50 ml. Samples 10 ml. were removed at intervals, and heated to inactivate the enzyme. The conversion into maltose was determined, and equal weights of polysaccharide (2.3 mg.) stained with iodine and water or (1.15 mg.) stained with iodine and ammonium sulphate solution.

FIGURE 11.

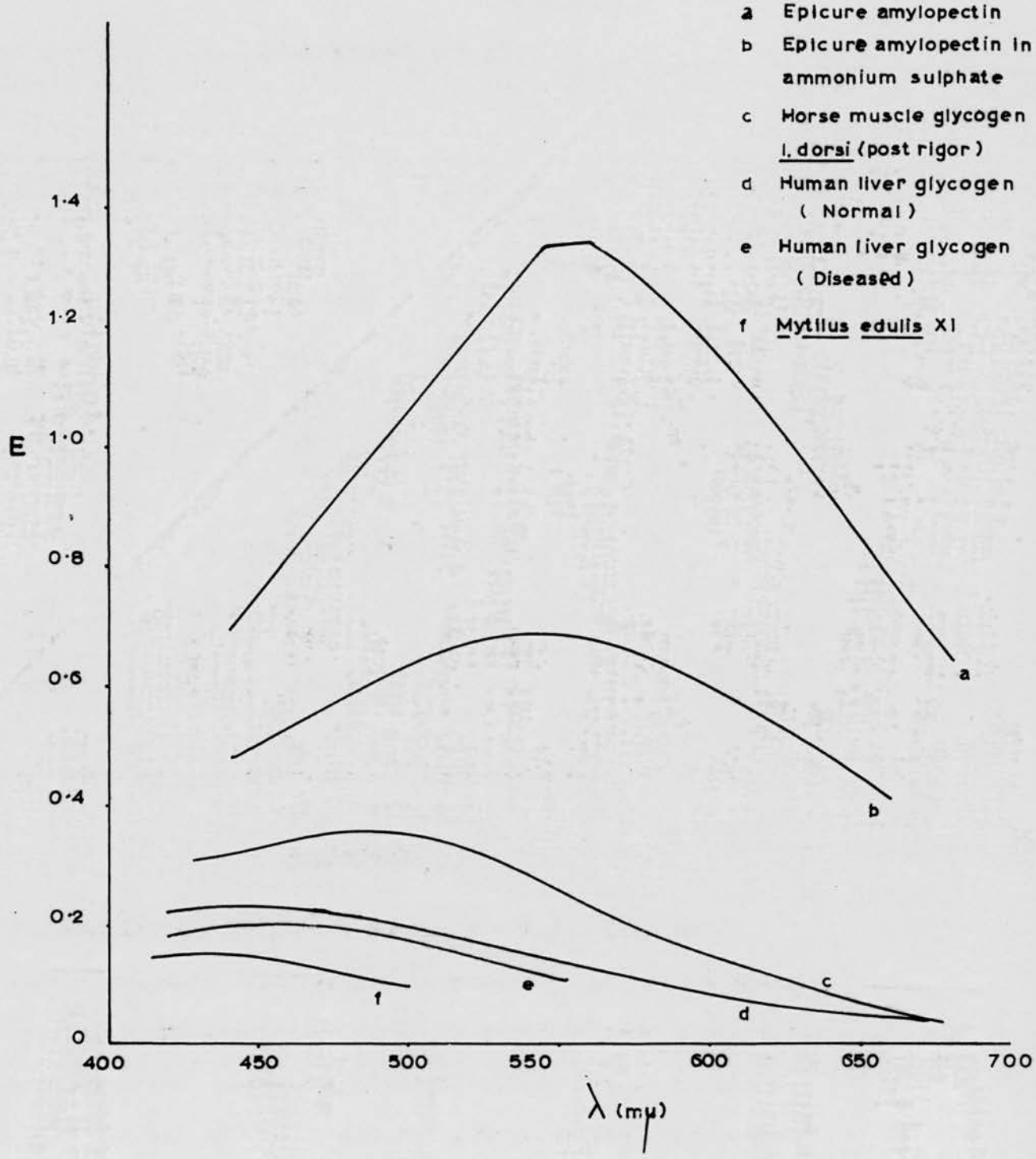

a Epicure amylopectin
b Epicure amylopectin in ammonium sulphate
c Horse muscle glycogen I. dorsi (post rigor)
d Human liver glycogen (Normal)
e Human liver glycogen (Diseased)
f Mytilus edulis XI
E
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
400
450
500
550
600
650
700
λ (mμ)
a
b
c
d
e
f

Table III. 18

Potato Amylopectin

Time of incubation (hr.)	β-Amylolysis limit (%)	Water		Ammonium sulphate	
		λ max (mμ)	E max	λ max (mμ)	E max
0	0	540	1.1	540	0.7
0.5	61	540	1.5	540	0.6
2.0	64	540	1.6	545	0.6
29	65	540	-	540	-
	Rabbit liver glycogen				
0	0	450	0.3	490	0.5
0.5	41	425	0.2	440	0.3
2.0	51	420	0.2	425	0.4
29	55	430	0.1	425	0.4

Discussion

Typical light absorption curves of the polysaccharide-iodine complexes are shown in Figure 11. With glycogens, a wide absorption peak covering 20-30 mμ was frequently obtained and the max values quoted represent the mid-points; with amylopectins a sharper peak was observed, and the λ max values are significant to ± 5 mμ .

The results in Tables III. 15 and 16 indicate a marked

difference in the absorption spectra of the iodine complexes of amylopectins and glycogens. The former complexes show much stronger absorption (λmax 515-550 mμ , E max 0.7-1.3) than those of glycogen (λmax 420-500 mμ , E max 0.1-0.5).

The iodine staining power of glycogens from different biological sources varies considerably, but there is no correlation between the λmax and E max and the average or exterior chain lengths. Generally, the iodine staining power increases in the order glycogen β-dextrin, invertebrate glycogen, mammalian liver glycogen, mammalian muscle glycogen. This same order was observed in Part 4 for increase of average exterior and interior chain lengths and suggests that the more compact the molecule the lower the iodine staining power.

In contrast to glycogens, amylopectins from different sources show similar λmax values but vary in the value of E max; this is attributed to the presence of small amounts of amylose impurity (135).

Removal of the exterior chains of glycogen with β-amylase (Tables III. 17 and 18) caused a decrease in the λmax and E max values. In contrast, amylopectin and its β-dextrin had the same λmax values. In amylopectin, therefore, the absorption of the iodine complex is not related to the length of the exterior chains. These results indicate that the type of iodine binding in the two polysaccharides is different.

It has been suggested that amylopectin binds iodine partly by adsorption of iodine molecules or triiodide ions and partly by the endwise axial arrangement of iodine molecules inside a series of

helices of α-1,4-linked glucose residues (136). Each coil of the helix is believed to contain six glucose residues and one iodine molecule and it is most probable that a minimum of three coils are necessary to produce a stain with iodine, i.e. an amylose type chain must contain 18 or more glucose units (137). Assuming equal numbers of A- and B-chains in both glycogen and amylopectin, the B-chain length is equal to 2 $\overline{CL}$ - $\overline{ECL}$. For an amylopectin of chain length 22 and a β-amylolysis limit of 58% the B-chain length is therefore 31 glucose units; KP amylopectin β-dextrin ($\overline{CL}$ 9.5) has a B-chain length of 16-17 glucose units. In contrast only a small number of the B-chains in glycogen and none in its β-dextrin would exceed $\overline{CL}$ 18.

Another factor which may cause differences in the type of iodine binding of the two polysaccharides is the very compact nature of the glycogen molecule especially on its surface as compared to amylopectin; as quoted previously (p.7) one particular glycogen sample was seven times more dense than an amylopectin molecule of the same size. This property may tend to prevent the penetration of iodine molecules and triiodide ions into the interior of the glycogen molecule. The spectral differences between the iodine complexes may therefore be related to internal branching characteristics.

Although the iodine absorption spectra of glycogen and amylopectin in water cannot be related to the P_R value, measurement of the λ max and E max in aqueous solution enables a distinction to be made between a glycogen and a amylopectin-type polysaccharide. In a qualitative experiment, a sample of Floridean starch supplied by

Dr. J.R. Turvey (sample T) was stained with iodine before and after degradation with β-amylase. Little difference (ca. 5 mμ) was observed in the λmax values indicating that Floridean starch, in this property at least, is an amylopectin-type polysaccharide.

Iodine staining in half-saturated ammonium sulphate

When glycogen is stained with iodine in the presence of half-saturated ammonium sulphate solution a rise in both λmax and E max occurs (Tables III. 16, 17 and 18). In contrast amylopectin and its β-amylase limit dextrin give a rise in E max only. Schlamowitz (138) has suggested that the ammonium sulphate facilitates iodine complexing by dehydration; this may provide a more hydrophobic environment in the helix coils of the B-chains for the iodine molecules. In the case of glycogen a limited amount of iodine-binding by the helical mechanism rather than by adsorption may then be possible. The increase of E max in the case of amylopectin is probably caused by additional adsorption suggesting that the helical mechanism largely determines the λmax.

Iodine staining under the conditions used in aqueous solution resulted in precipitation of the polysaccharide-iodine complex. In one experiment, E max fell from 0.87 to 0.82 in 15 minutes. The polysaccharide concentration was therefore halved, thus increasing the stability of the iodine complex. E max values were halved as a result, but the λmax remained unchanged, a slightly sharper peak being obtained.

Manners and Archibald (135) have shown that the λmax in half-saturated ammonium sulphate solution is approximately related to the degree of branching by the equation:

$$\overline{CL} = 16 + 0.114 (\lambda max - 500)$$

The standard error in a chain length determination is ca. 1.6 glucose residues. The values for both amylopectin and glycogen in Tables III. 15 and 16 are in good agreement with this equation and indicate that iodine staining under these conditions may enable the degree of branching of micro quantities of a polysaccharide to be determined.

Part 6 Reaction with Concanavalin-A

Introduction

The reaction between glycogen and the globulin concanavalin-A was first described by Summer and Howell (139). Cifonelli and Smith (111) developed a method for the turbidimetric measurement of this protein-polysaccharide reaction, which has been used for the determination of 'glycogen values' for glycogens from a variety of sources. The glycogen value varied with the source and with the degree of branching, as β-amylase limit dextrins gave higher values than the original polysaccharides. Neither amylose, amylopectin, laminarin nor dextran gave any reaction with concanavalin-A (112).

The above workers concluded that the reaction was connected with the interior of the molecule.

In the present study concanavalin-A has been prepared and its reaction with glycogens and other α-1,4-glucosans studied.

Results

The concanavalin-A reagent was prepared and used as in Section 2. Polysaccharide concentrations were always determined by complete acid hydrolysis and estimation of the liberated glucose.

In earlier experiments turbidimetric measurement at three polysaccharide concentrations was carried out, the optical density given by 1 mg. being obtained from a graph of optical density versus concentration. Individual values especially in the 400-700 μ g. range were found to give similar results, thus in later experiments single concentrations in this range were used.

The optical density was found to reach a maximum after 10 minutes and usually remained constant for a further 20 minutes. The greater the optical density value, the more unstable the solutions were found to be.

Table III. 19

Source of glycogen	Concentration (μg.)	Maximum Optical Density	Optical density (1000 μg.)	G.V.	$\overline{ECL}$*
Rabbit liver (standard)	400 600 800	0.109 0.165 0.222	0.278	1.00	-
Helix pomatia	195 486 780	0.074 0.170 0.280	0.356	1.28	4
Tetrahymena pyriformis	149 372 596	0.046 0.124 0.199	0.331	1.19	8-9
Rabbit liver (IX)	194 484 776	0.064 0.158 0.249	0.321	1.16	7-8
Foetal pig liver	201 503 804	0.062 0.162 0.254	0.315	1.13	8
Trichomonas foetus	193 482 772	0.060 0.148 0.238	0.305	1.10	11-12

* Exterior chain length

Table III. 20

Source	G.V.	$\overline{ECL}$
Horse psoas (post-rigor)	0.76	10
Ox sternocephalicus (pre-rigor)	0.78	12
Horse diaphragm (pre-rigor)	0.81	11-12
Ram liver	0.82	9
Horse l.dorsi (pre-rigor)	0.82	11-12
Horse heart (post-rigor)	0.83	10-11

Table III. 20 continued

Source	G.V.	ECL
Horse diaphragm (post-rigor)	0.85	11
Ox psoas (pre-rigor)	0.86	10-11
Horse l.dorsi (post-rigor)	0.87	10
Pig liver (sugar fed)	0.87	11
Ox sternocephalicus (post-rigor)	0.89	9
Rabbit liver VII	0.97	9-10
Yeast	1.14	8
β-Amylase dextrins		
Oyster	1.76	2.5
Mytilus edulis XI	1.62	2.5
Ram liver	1.41	2.5
Foetal sheep liver* (alkali treated)	1.39	2.5
Rabbit liver VII	1.38	2.5
Foetal sheep liver	1.18	2.5
Rabbit muscle III	1.14	2.5
Rabbit liver LD1 **	0.99	5
Plant Glycogens (Zea mays polysaccharides)*		
Phytoglycogen A	0.09	9*
Phytoglycogen B	0.96	5*
Fractions 55-60	0.78	-
60-65	0.75	-
65-70	0.74	-
Floridean starches		
Floridean starch (I)**	0.08	
Floridean starch (sample T)	0.17	
Floridean starch (II)**	1.40	
Starches and starch components		
Soluble starch	0.00	
Nitella translucens starch *	0.00	

Table III. 20 continued

Source	G.V.
Starches and starch components	
KP Amylose	0.00
Waxy maize starch (I)	0.00
KP Amylopectin	0.00
D. bioculata amylopectin	0.00
Maize amylopectin	0.00

⁂ Liddle and Manners (140)

⁂ See Bell and Manners (141)

* See Peat, Whelan and Turvey (142)

⁂ Fleming, Hirst and Manners (143)

⁂ Provided by Mr. N.J. King

Periodate oxidised samples

Periodate oxidised samples of glycogen were tested with the concanavalin-A reagent. The oxidation mixtures (*ca.* 2 mg./ml. glycogen; 4 mg./ml. amylopectin) were used directly, after addition of ethylene glycol and adjustment to pH 6.0. Samples (1 ml.) were added to concanavalin-A in the normal way and read against a blank which was prepared by addition of a periodate control (1 ml.) containing no polysaccharide. Three glycogens and two amylopectins examined in this way gave no reaction. On addition of a few drops of glycogen to each of the solutions a turbidity was obtained.

Effect of high polysaccharide concentration on the reaction

Addition of glycogen in excess of 1 mg. gave turbid solutions followed fairly rapidly, depending on the quantity, by precipitation. Amylopectins gave unusual results when added in large quantity, for example, when KP amylopectin (1 ml.; 10 mg./ml.) was added to concanavalin-A the following results, measured on an EEL colorimeter using a No.422 light filter, were obtained:

Time (minutes)	Optical density
3	0.98
6	0.41
9	0.35
15	0.10
20	0.00

The solution became turbid immediately after addition of the amylopectin but no precipitation occurred. The optical density given, after 10 minutes by a glycogen (0.514mg;G.V. 0.97) under the same conditions was 0.390.

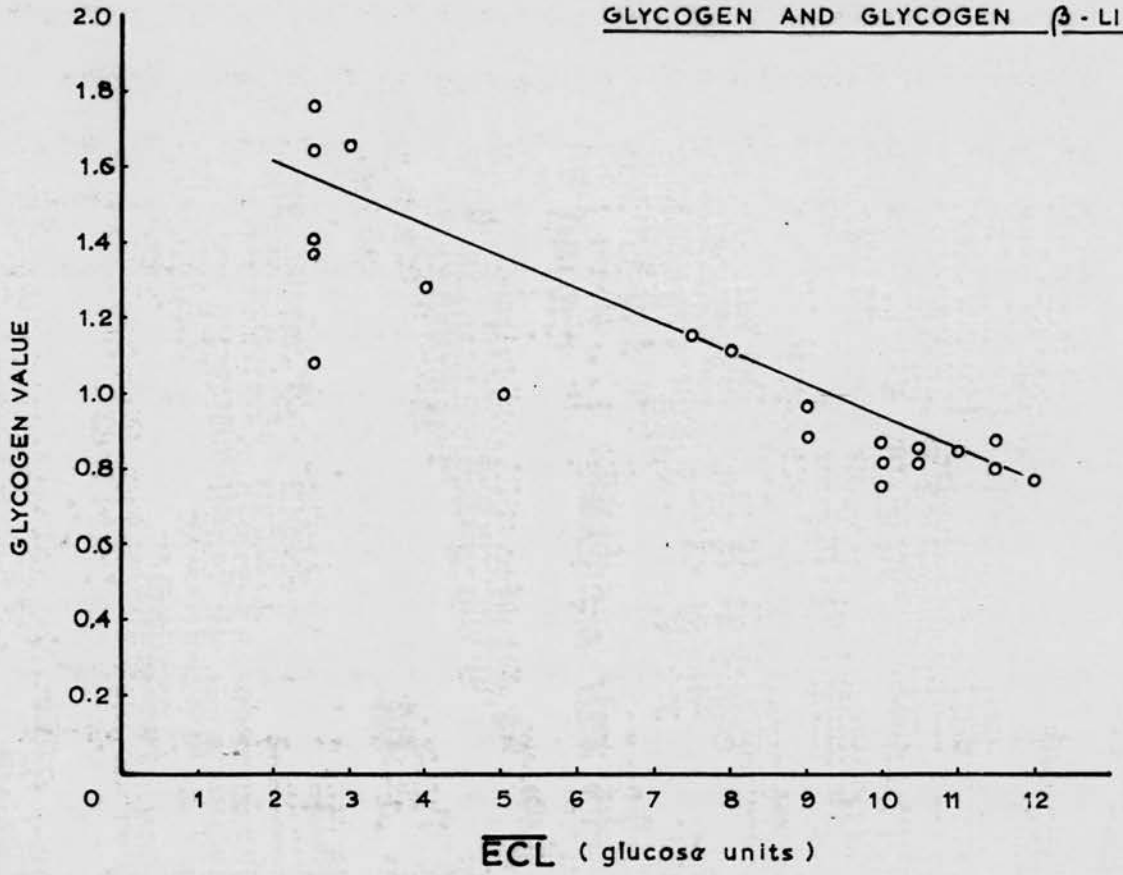
Under these conditions KP amylopectin β-dextrin (5 mg.) decreased from a value of 1.65 to zero in 20 minutes. Excess of maize amylopectin gave similar results.

Discussion

Most glycogens with normal branching characteristics have glycogen values in the region of 1.0; those with a low degree of branching such as muscle glycogens, tend to have lower values than this, and those with a higher degree of branching such as the glycogen

FIGURE 12

RELATIONSHIP BETWEEN THE GLYCOGEN VALUE AND THE EXTERIOR CHAIN LENGTH OF GLYCOGEN AND GLYCOGEN β-LIMIT DEXTRIN



from Helix pomatia and the β-amylase dextrins have values greater than 1.0.

A graph of glycogen value versus exterior chain length (Figure 12) shows that the glycogen value is to some degree controlled by this latter property, long exterior chains hindering the polysaccharide-protein reaction. Generally, the glycogen value increases with decrease of exterior chain length. However, the fact that neither amylopectin nor its β-limit dextrin react with concanavalin-A suggests that the interior branching characteristics of the molecules also have a marked effect on the reaction.

After prolonged periodate oxidation, glycogens lose their ability to react with concanavalin-A in accord with the results of Smith and coworkers who found that glycogen value decreased with increasing time of oxidation. This suggests that the hydroxyl groups of the polysaccharide play a part in the reaction also.

The results obtained for phytoglycogen fractions A and B are unusual; the former high molecular weight fraction gave practically no reaction with concanavalin-A, while the latter had G.V. 0.97. These polysaccharides were obtained by Dr. J.R. Turvey by fractionation of the iodine staining material in the cell sap of Zea mays with glacial acetic acid; both samples gave brown iodine stains and had β-amylolysis limits of ca. 45%.

The non-reaction of fraction A could be due to either its molecular size or to its internal branching characteristics which might be of the amylopectin rather than the glycogen type, i.e. ICL 6.

If molecular size is a controlling factor, this might be a partial explanation as to why amylopectins do not react with concanavalin-A since it has been reported that many amylopectins have molecular weights of 300 million and greater (30).

In a qualitative experiment, horse muscle glycogen (ca. 1 mg.) of molecular weight 60 million (from light scattering measurements) provided by Dr. C.T. Greenwood, reacted normally with concanavalin-A, higher concentration causing precipitation. An amylopectin-type polysaccharide extracted from Caulerpa filiformis (ca. 1 mg.) kindly provided by Dr. I.M. Mackie, gave no reaction with concanavalin-A; this polysaccharide had a molecular weight of 15,000 which is much less than that of many glycogens (144). It therefore seems unlikely that molecular size is the factor which controls reactivity. However, it seems equally unlikely that phytoglycogen A has the internal branching characteristics of an amylopectin since it has a glycogen-type iodine stain. Addition of excess of fraction A (2-3 mg.) to concanavalin-A produced a fairly turbid solution, and after 10-15 minutes precipitation occurred which is typical of glycogen. This polysaccharide is most probably of the glycogen-type but for a reason, which has not yet been discovered, gives a very low glycogen value under standard conditions.

Two of the Floridean starch samples, one provided by Dr. J.R. Turvey and sample I gave practically no reaction with concanavalin-A while sample II reacted strongly (G.V. 1.40). The Floridean starch containing alga, Dilsea edulis, also contains galactan

sulphates. Concanavalin-A will also react with heparin and mucoitin (145) and would probably react with traces of galactan sulphates in sample II; this might explain the high glycogen value. Galactose was detected in an acid hydrolysate of this sample by descending paper chromatography. The very low glycogen values of the other samples, together with their reddish-purple iodine stains suggests that Floridean starch is an amylopectin-type polysaccharide.

Concanavalin-A could be used as a sensitive test for the detection of impurities in certain polysaccharides, e.g. the presence of galactan sulphate in Floridean starch. It should also prove useful during the fractionation of the iodine staining polysaccharides in the cell sap of Zea mays, and in the micro-analysis of glycogen in metabolic experiments.

The results obtained with high concentrations of amylopectin are probably due to the polysaccharide acting as a protective colloid, although in such a case there should be no initial turbidity. Cifonelli and Smith (111) observed no reaction between yeast mannan (20 mg./ml.) and concanavalin-A but at normal concentrations (1 mg./ml.) the mannan gave a higher glycogen value than rabbit liver glycogen. In the case of amylopectin, there might be an initial reaction, gradually suppressed by the high polysaccharide concentration.

Part 7 Glycogen Structure and Rigor Mortis

Introduction

Glycogens from different sources and even from the same source can vary with respect to such properties as molecular weight, average chain length and the relative lengths of exterior and interior chains. That the molecular inhomogeneity of glycogens is of metabolic significance has been demonstrated by Stetten, Katzen and Stetten (146) who have shown that in rat and rabbit muscle the largest molecules are the most reactive, and by Stetten and Stetten (134) who have shown that the glucose residues at the periphery of the molecule have a higher rate of turnover than those in the centre.

During post-mortem glycolysis, which is accompanied by the onset of rigor mortis, the pH in mammalian muscles drops to 5.4 - 5.5 at which point the lactic acid producing enzyme systems are inactivated. At this pH, provided pre-slaughter stress has not been too great, some glycogen may still remain. At a final pH of 5.9, 0.6-1% of glycogen frequently remains in the psoas and diaphragm of the horse (147) and 0.6% at a final pH of 6.1 in the sternocephalicus muscle of the ox (148). This aspect of the apparent physiological inhomogeneity of glycogen has been investigated in the present work. The residual glycogen, remaining after the onset of rigor mortis, could either be inaccessible or insusceptible to enzymic attack or it could be structurally different from the glycogen in the pre-rigor state. A structural analysis of glycogens prepared from horse and ox muscles in the pre- and post-rigor states has been carried out to investigate these possibilities.

Results

The results of analyses mentioned in previous parts of this Sectinn are summarised in Table III.21.

Table III. 21

Properties of Horse and Ox Muscle Glycogens

Sample		Chain length (average)	Glycogen value	λ max (mμ)	E max	P_M	β-Amylolysis limit (%)	$\overline{ECL}$
Horse, l.dorsi	pre-rigor	17	0.87	480	0.34	83	53	11-12
	post-rigor	17	0.82	490	0.36	78	47	10
Horse, diaphragm	pre-rigor	17	0.81	475	0.31	80	53	11-12
	post-rigor	17	0.85	480	0.32	80	51	11
Horse, psoas	pre-rigor	16-17	0.86	490	0.32	79	48	10-11
	post-rigor	17	0.76	490	0.35	76	46	10
Horse heart,	post-rigor	16-17	0.83	470	0.29	78	48	10-11
Ox, psoas	pre-rigor	16-17	0.86	490	0.34	82	50	11
	post-rigor	15	-	480	0.30	79	50	10
Ox, sternocephalicus	pre-rigor	19	0.78	475	0.27	83	51	12
	post-rigor	15	0.89	485	0.21	79	44	9

Discussion

Previous Studies on Muscle Glycogen

Relatively few muscle glycogen samples have been studied probably because of the low glycogen content of muscle and the difficulty of extraction compared to liver and other sources. The rabbit muscle glycogen III studied in this work has an average chain length of 13 glucose residues and a β-amylolysis limit of 46%, properties which are similar to those obtained for several rabbit liver samples. Other workers have reported average chain length values of 11-13 and β-amylolysis limits of 42 ± 4%, for muscle glycogens. A number of results are summarized in Table III. 22.

Table III. 22

Properties of mammalian-muscle glycogens

Sample	Average chain length	β-amylolysis limit (%)	$\overline{ECL}$	Ref.
Horse	11-12 (m,p)	42	7	(149,125)
Human	12 (p)	41	7	(125)
	11 (p)	40	7	(140)
Rabbit	11-13 (m,p)	45	8	(150,125)
	11 (p)	39	7	(140)
	15 (e)	-	-	(27)

Methods of assay are indicated by : (m) methylation; (p) periodate oxidation; (e) enzymic.

The chain length values in Table III.22 resemble the greatest majority of values reported for liver and invertebrate

glycogens (140, 127).

Structure of Horse and Ox Muscle Glycogens

The most noticeable feature of the results recorded in Table III. 21 is that the average chain length values are much higher for the horse and ox muscle samples than those reported in the present work for glycogens from diseased and normal liver, from invertebrate glycogens and from those of previously reported muscle glycogens. These results were obtained using an established method of periodate oxidation so that there is little chance that the unusually high values could be due to experimental error. Also, the horse l.dorsi muscle glycogen was oxidised several times under the same conditions as liver and invertebrate glycogen controls, the same result being repeatedly obtained. The value of 18.6 glucose residues for the ox sternocephalicus (pre-rigor) glycogen was obtained in a second estimation, the first having given a value of 19.0. The glycogens therefore have a lower degree of branching than normal (i.e. low P_R values).

The β-amylolysis limits, which are higher than normal except in the case of ox sternocephalicus (post-rigor) glycogen, indicate long exterior chain lengths of 10-12 glucose residues compared to 7-8 for the muscle glycogens in Table III. 22.

These results are supported by the P_M values which give calculated chain lengths in support of a low degree of branching, and by the glycogen values which are low due to the length of the exterior chains, which hinder the reaction.

The iodine binding power is greater than that of liver and invertebrate glycogens. This property is not however related to molecular structure (135) but is characteristic of muslce glycogens (151). This property serves to characterize the muscle polysaccharides as glycogens rather than amylopectins. For example, the β-amylase limit dextrin of waxy maixe starch I has an average chain length of 9 but has λ max 535 mμ and E max 0.9.

The results in Table III.21 show that the properties of the pre- and post-rigor samples from horse muscles and ox psoas are not significantly different. In ox sternocephalicus there is a significant shortening of the exterior chains from 12 to 9 during post mortem glycolysis.

Although the pre- and post-rigor glycogens from horse muscles and ox psoas have similar branching characteristics they could be different with respect to molecular weight since different molecular weight fractions of glycogen can have the same branching characteristics (121). Since the large molecules of muscle glycogens are metabolised more rapidly than the small ones by muscle phosphorylase (146) it is probable that the post-rigor samples would have a lower molecular weight average than those from the pre-rigor state. Dr. W.A.J. Bryce determined the sedimentation constants of the l.dorsi muscle glycogens; values of 96 and 80 x 10^{13} c.g.s. unit were obtained for the major components of the pre- and post-rigor samples, corresponding to molecular weights of 4.4 and 3.7 x 10^{6} (assuming a diffusion constant of 1.5 x 10^{-7}). Although the difference is small, these results do agree with the above suggestion. The

work of Stetten and coworkers, mentioned above, was mainly carried out on TCA extracted glycogen. It would be of interest to obtain pre- and post-rigor glycogens by TCA extraction and compare the difference in molecular weight with the above results.

Since the degree of branching is similar in the pre- and post-rigor glycogens from horse muscle and ox psoas, the relative activities of the enzymes involved in glycogen breakdown (i.e. phosphorylase, α-glycosyltransferase and amylo-1,6-glucosidase) must remain the same, as alteration in activity would produce glycogen with short exterior chains, assuming that phosphorylase must be active to catalyze glycogenolysis. A decrease in the activity of the debranching enzyme system or *in vivo* heterogeneity of the glycogen in ox sternocephalicus muscle would explain the decrease from 12 to 9 glucose residues in the exterior length.

Summary

1. The average chain lengths of glycogens and amylopectins have been measured by potassium and sodium periodate oxidation. A sodium periodate method, which could be used on the 50-100 mg. scale has been investigated; a rapid linear overoxidation occurred which gave values, by extrapolation to zero time, in good agreement with those obtained by the potassium method. Oxidation of trehalose by this method gave a maximum production of 1.15 moles per non-reducing end-group. It is suggested that similar overoxidation of all dialdehyde residues in the polysaccharide molecules may take

place, and account for the additional production of formic acid. Other conditions of sodium periodate oxidation have been examined.

2. A quantitative relationship has been found to exist between the α-amylolysis limit and the degree of branching of glycogens, enabling the average chain length to be calculated from measurement of the former property. A modified technique for α-amylolysis, involving an initial β-amylolysis, gave a more accurate indication of the degree of branching in both glycogens and amylopectins than direct measurement of the α-amylolysis limit. It is suggested that this method may be advantageously used in the semi-micro examination of amylopectin-glycogen type polysaccharides.

3. The β-amylolysis limit has been used for the calculation of $\overline{ECL}$ and $\overline{ICL}$ values from the average chain length of glycogens and amylopectins. Both values were found to increase in the order: invertebrate glycogens, liver glycogens, muscle glycogens, and amylopectins.

4. The absorption spectra of glycogen-iodine complexes in water were found to be significantly different from those of amylopectin. Removal of the exterior chains of these polysaccharides by β-amylase caused a decrease in the λmax and E max values of glycogens; no decrease in λ max was observed in the case of amylopectins, indicating that the type of iodine binding in the two polysaccharides is different.

In presence of half-saturated ammonium sulphate, λ max and E max values of glycogens increased; under these conditions the spectra of amylopectin-iodine complexes were affected to a much lesser extent. Values of λ max of amylopectin and glycogen complexes in half-saturated ammonium sulphate were proportional to the $\overline{CL}$ values of the polysaccharides.

5. The globulin concanavalin-A has been used for the determination of glycogen values of glycogen-type polysaccharides; the intensity of the reaction was found to be inversely proportional to the $\overline{ECL}$ value, long exterior chains hindering the reaction. Amylopectins and amylopectin β-limit dextrins gave no reaction with concanavalin-A which indicates that the internal branching characteristics of the molecule may control the reaction. It is suggested that measurement of glycogen value may be used to distinguish between amylopectin and glycogen-type polysaccharides.

6. The molecular structure of a series of mammalian muscle glycogens isolated during the pre- and post- phases of rigor mortis has been studied by chemical and enzymic methods. The glycogens had $\overline{CL}$ values of approximately 17 which indicates a lower degree of branching than has been previously reported for those isolated from mammalian liver or muscle.

Glycogen isolated from ox sternocephalicus muscle after the onset of rigor mortis was found to have shorter exterior chains than that isolated from the pre-rigor muscle, indicating partial inactivation of the glycogenesis-glycogenolysis system or a true heterogeneity of the glycogen in this muscle in vivo.

SECTION IV

STUDIES ON GLYCOGEN STORAGE DISEASE

INTRODUCTION

Glycogen storage disease was first reported in 1929 by von Gierke (152) as a condition in which excess glycogen accumulates in the liver and kidney cortex but not in other tissues. In a survey of a number of cases, Cori (153) has suggested the existence of four types of the disease. In type 1. (von Gierke's disease) the glycogen has a normal physical and chemical structure and the disease is characterized by a low level of activity of glucose-6-phosphatase. In type 2. disease, (generalized glycogenosis), all the tissues which normally synthesize glycogen contain it in large amount. As in type 1. the structure and properties of the glycogen are normal but the biochemical abnormality has not yet been discovered. Types 3. and 4. differ from the first two in that the glycogen is of abnormal structure. In type 3. the glycogen which accumulates in the muscles, in the heart, and in the liver has unusually short exterior chains, the enzymic lesion probably being an absence of amylo-1,6-glucosidase. The glycogen of type 4. disease is less branched than normal glycogen and has longer exterior and interior chains. This polysaccharide resembles amylopectin in these properties and also in solubility and absorption spectrum of the iodine complex.

The enzyme deficiency is presumably a low level of amylo-(1,4→1,6)-transglucosidase (branching enzyme).

A further type of disease has been reported by McArdle (154) and more recently by Mommaerts and coworkers (90). This disease, which is confined to skeletal muscle, is characterized by the absence of phosphorylase which results in the storage of glycogen synthesized by the UDPG pathway (Figure 4). The glycogen content reported was about 4% which is low compared to that of type 2. disease. In the latter, the glycogen content can be as much as 12%, although phosphorylase activity is normal. This fact has led to the suggestion that there are other regulatory factors which influence the amount of glycogen deposited in muscle. This disease could be called type 5. disease. The various types of glycogenosis are summarized below:-

Type	Enzymic Lesion	Structure	Storage sites
1	Glucose-6-phosphatase	Normal	Liver and kidney
2	-	Normal	Generalized
3	Amylo-1,6-glucosidase	Abnormal	Heart, muscle, liver, kidney
4	Amylo-(1,4→1,6)-transglucosidase	Abnormal	Liver
5	Phosphorylase	Normal	Skeletal muscle

Two lines of approach have been used in characterization of diseases of this type; (a) structural analysis of the isolated polysaccharide, and (b) determination of enzyme activity levels.

A combination of the two methods is to be preferred. The present studies are concerned with the structural analysis of the glycogens from four cases of the disease.

Case I

Additional properties of the liver glycogen from a fatal case of glycogen storage disease, investigated earlier by Manners (155), have been carried out. In this case the enzymic lesion was probably an absence of amylo-1,6-glucosidase (type 3. disease).

Case II

Samples of tissue from the liver, kidney, brain and striated muscle of a fatal case were provided by Sir Hans Krebs, F.R.S. The patient (A.K.) was a sibling of the patient (S.K.) in Case I.

Case III

Diseased tissue from a fatal case and normal liver tissue as a control were provided by Dr. P.W. Kent.

Case IV

A biopsy sample of liver tissue was obtained from Dr. S. Bain, Pathology Department, Sick Children's Hospital, Edinburgh.

Experimental

Extraction and Purification of Glycogens

Case II. The boiled liver tissue (243 g.) was extracted with hot water, purified and dried as described in Section 3. Yield, 18.9 g. (7.8% yield from the tissue). Glycogen was extracted in a similar way from the boiled kidney tissue (78 g.). Yield, 3.4 g. (4.4% yield from the tissue). Further extraction of the liver and kidney residues with 30% potassium hydroxide at 100° did not yield any additional glycogen.

No glycogen could be isolated from the boiled brain or muscle tissues which were successively extracted with water and 30% potassium hydroxide at 100°.

Case III. The boiled liver tissue (69 g.) was homogenized with cold water to give a fine suspension which was centrifuged. Glycogen was obtained from the cold aqueous extract by precipitation with ethanol (3 vol.) after deproteinization in the usual way. It was purified and dried as before. Yield, 1.36 g. (Fraction Dc). The tissue residue on hot water extraction gave 2.03 g. glycogen (Fraction Dh). The total yield was 4.9% of the tissue weight.

The control sample of normal liver tissue (51 g.) was extracted in the same way, the yields being 400 mg. (Fraction Nc) and 153 mg. (Fraction Nh) respectively for the cold and hot aqueous extracts, equivalent to a total yield of 1.1% of the tissue weight.

Further extraction of the aqueous extracted residues with 30% potassium hydroxide gave no glycogen.

Glycogen Content of Tissues

The method was essentially that of Good, Kramer and Somogyi

(156). The diseased liver tissue (588 mg.) was digested with 30% potassium hydroxide (1.5 ml.) at 100° for 20 minutes. Saturated sodium sulphate solution (0.25 ml.) was added and the glycogen precipitated by the addition of ethanol (1.2 vol.) with stirring. After centrifugation, the glycogen was redissolved in water (ca. 1 ml.) and reprecipitated with ethanol (1.2 vol.). This procedure was repeated, the last traces of ethanol being removed by heating the glycogen precipitate on a boiling water bath. On acid hydrolysis 61.1 mg. of glucose was produced equivalent to a glycogen content of 9.4%. By a similar analysis, the normal liver (530 mg.) had a glycogen content of 1.8%.

Case IV (Biopsy material). The tissue sample 233 mg.) was digested with 30% potassium hydroxide (1.5 ml.) at 98° for 20 minutes. The resulting pale brown solution was centrifuged, saturated sodium sulphate (0.2 ml.) was added to the supernatant solution, and the glycogen precipitated by addition of ethanol (2 ml.; 1-1.2 vol.). It was purified by seven further precipitations. The whole procedure was carried out in a single graduated centrifuge tube.

The glycogen was kept in solution (6.4 ml.). Yield, 34 mg. by acid hydrolysis of an aliquot, i.e. 14.6% of the tissue weight.

Characterization of the Purified Glycogens

Case II

1. Complete Acid Hydrolysis

On hydrolysis the liver glycogen (24.6 mg.) gave glucose (26.2 mg.) equivalent to 96% glucosan, and no other sugar; the kidney

glycogen (24.2 mg.) gave glucose (26.4 mg.) equivalent to 98% glucosan.

2. Specific Rotation

Liver glycogen (55.5 mg.) was dissolved in water (25 ml.) and the optical rotation, measured in a 2 dm. tube, was + 0.87° equivalent to $[\alpha]_D$ + 197°. The kidney glycogen (56.3 mg.) gave an optical rotation of + 0.90° equivalent to $[\alpha]_D$ + 200°.

3. Absorption Spectra of Iodine Complexes

The liver glycogen stained a deep red-brown in aqueous solution (λmax (H_2O) 460-465 mμ ; E max 0.21), the colour becoming much deeper in half-saturated ammonium sulphate (λmax ($AmSO_4$) 480-490 mμ ; E max 0.51). The iodine complex of the kidney glycogen gave; λmax (H_2O) 445-450 mμ ; E max 0.19; λmax ($AmSO_4$) 460-465 mμ ; E max 0.62.

4. Periodate Oxidation

Liver glycogen (262.2 mg.) gave 5.06, 5.11 and 5.11 mg. of formic acid after oxidation with potassium metaperiodate at room temperature for 9, 14 and 16 days respectively, the latter value corresponding to an average chain length of 14.6 glucose residues. In a duplicate experiment, 261.0 mg. of liver glycogen gave 5.17 mg. of formic acid on complete oxidation which corresponds to an average chain length of 14.5. In duplicate estimations the kidney glycogen (253.7 mg.) gave, under similar conditions, a final production of 5.06 mg. and 5.07 mg. respectively, after 17 days, which corresponds to average chain lengths of 14.3 and 14.1.

Liver glycogen (100.30 mg.) in water (22 ml.) was also oxidised at 2° in the dark with 3 ml. of 8% sodium metaperiodate. The production of formic acid released after oxidation for 10, 14 and 20 days was 1.79, 1.92 and 1.98 mg. respectively, the last value corresponding to an average chain length of 14.4.

Liver glycogen (95.2 mg.) was also oxidised by the method of Perlin (131) giving the following results:

Table IV (1)

Time (hr.)	Sodium hydroxide titre (ml.)	Formic acid (%)	Average chain length
50	0.96	2.16	13.13
97	1.04	2.34	12.12
146	1.14	2.57	11.03
191	1.27	2.84	10.00

The chain length obtained by extrapolation to zero time is 14.2 glucose residues.

5. Glycogen Values

The measurement of the optical density of 1 mg. of the AK glycogens in the presence of concanavalin-A gave glycogen values of 0.97 and 1.01 for the liver and kidney samples, respectively.

6. Enzymic Degradation

(a) α-Amylolysis

The results of the salivary α-amylolysis of the AK liver

and kidney glycogens together with those from control samples of rabbit liver glycogen (VII), and potato amylopectin (KP) are shown in the following Table:

Table IV (2)

Time of incubation (hr.)	2.5	5	24
		P_M value	
Liver glycogen (A.K.)	62	66	74
Kidney glycogen (A.K,)	63	67	76
Rabbit liver glycogen	65	69	75
Potato amylopectin	74	79	89

(b) β-Amylolysis

On treatment with barley β-amylase at pH 4.6 the AK liver (26.2 mg.) and kidney (27.2 mg.) glycogens gave 12.6 mg. and 13.1 mg. maltose respectively, after 24 hr. incubation, equivalent to a β-amylolysis limit of 46% in both cases.

(c) β-Amylolysis after pre-treatment with isoamylase

The AK liver and kidney glycogens, and KP amylopectin were incubated with isoamylase (50 mg.), extracted from Brewer's yeast by Dr. Z.H. Gunja, at pH 5.9 for 24 hr. at 20°. The enzyme was inactivated by heating; 5 ml. of 0.2 M acetate buffer pH 4.6, β-amylase (2000 units) and water to 25 ml. were then added. After a further 24 hr. the maltose production was measured giving the following results:-

FIGURE 13

LIMITING VISCOSITIES OF SOME POLYSACCHARIDES

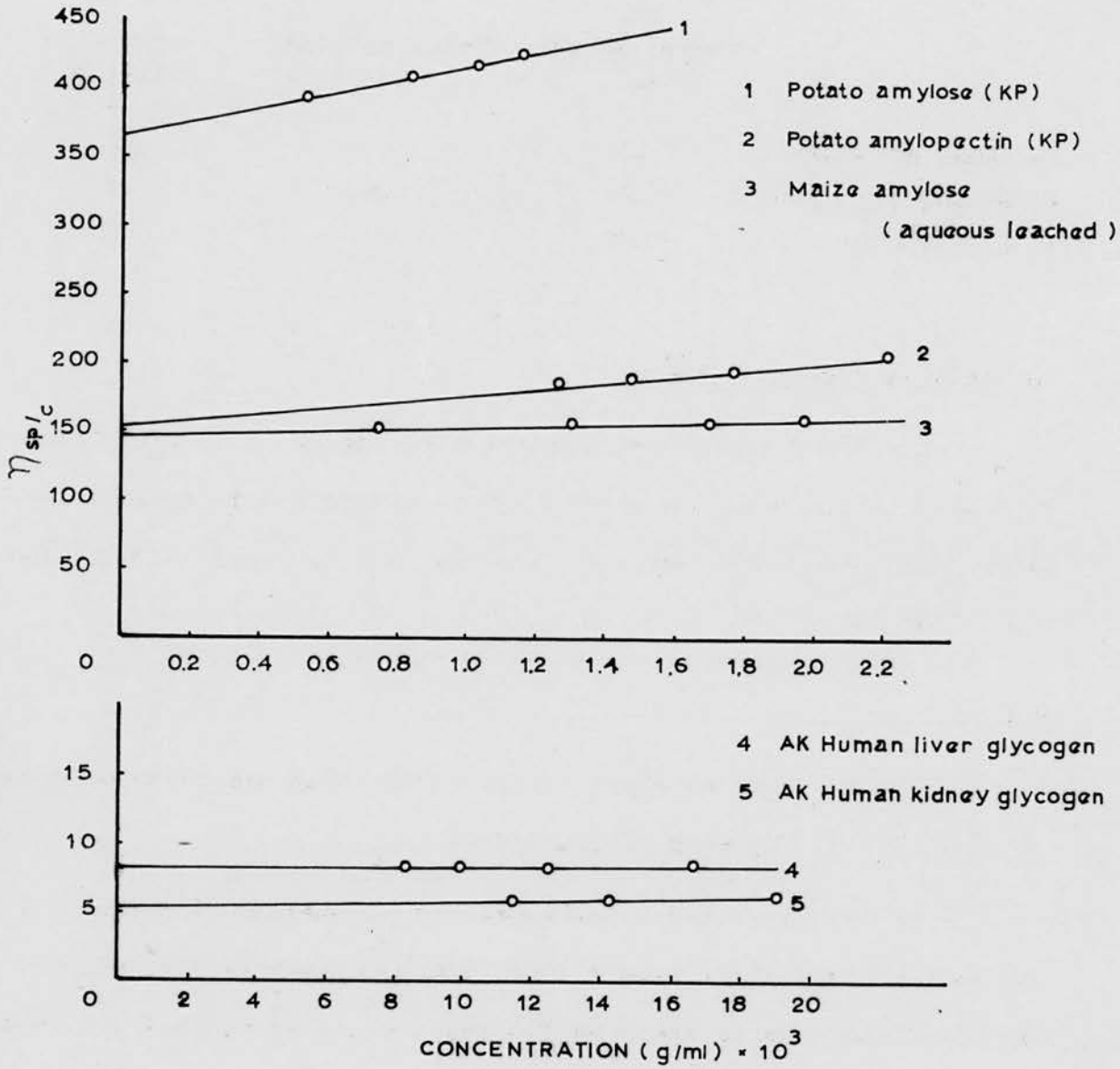


Table IV (3)

	Sample weight (mg.)	Total maltose (mg.)	β-Amylolysis limit (%)
AK liver glycogen	27.1	17.3	60
AK kidney glycogen	26.0	16.3	59
KP amylopectin	27.3	21.3	77

7. Limiting Viscosity Number

The limiting viscosity numbers of the AK liver and kidney glycogens in 0.1 M sodium chloride were determined using dilution method 1. The values obtained were 8.3 and 5.3 respectively (See Figure 13).

Case I

The SK liver glycogen was examined using the methods which were applied to the Case II glycogens.

On sodium periodate oxidation by the method of Perlin (131) the extrapolated chain length value was 6.3 (Section III, Part 2) (By potassium metaperiodate oxidation, a value of 6.3 had previously been obtained).

The optical density given by 1 mg. in presence of concanavalin-A at 420 mμ was 0.434, giving a glycogen value of 1.61.

Salivary α-amylolysis gave P_M values of 24, 27 and 33% after 2.5, 5 and 24 hr. respectively.

β-Amylolysis of the SK glycogen (29.1 mg.), pre-incubated

with isoamylase (50 mg.), gave 20% conversion into maltose. The glycogen had a limiting viscosity of 10.6 in 0.1 M sodium chloride and 11.3 in water.

Case III.

1. Complete acid hydrolysis

Glucose was the only sugar which could be detected in hydrolysates of the normal and diseased glycogens, the following glucose contents being obtained: Nc 101%, Nh 92%, Dc 102%, D_h 98%.

2. Absorption spectra of the iodine complexes

The iodine complexes of the glycogens gave the following results: Nc: λmax (H_2O) 430 mμ, E max 0.302; N_h: λmax (H_2O) 450 mμ, E max 0.259; Dc: λmax (H_2O) 440-445 mμ, E max 0.265; D_h: λmax 435 mμ, E max 0.203.

3. Periodate Oxidation

The method of Perlin (131) was used for the oxidation of the diseased liver glycogens (Dc, 97.4 mg.; D_h, 91.0 mg.) and the cold water extracted normal liver glycogen (60.4 mg.) giving the following results:-

Table IV (4)

Time (hr.)	Apparent chain length values (Glucose units)		
	Nc	Dc	D_h
48	14.7	13.4	13.8
92	14.1	12.3	12.7
122	13.6	-	-
147	13.5	10.7	11.3

Extrapolation of the graph of chain length versus time to zero time gives values of 15.2, 14.7 and 15.1 for Nc, Dc and D_h samples respectively.

4. Glycogen Values

Samples Nc and Dc reacted with concanavalin-A giving glycogen values of 0.88 and 0.92 respectively using AK liver glycogen as a control.

5. Enzymic Degradation

(a) α-Amylolysis

The results of the action of salivary α-amylase on the glycogens with AK liver glycogen as a control, are shown in the following Table:

Table IV (5)

Time of incubation (hr.)	5.25	6.4	26
		P_M values	
Nc	76	76	81
N_h	75	77	79
Dc	74	75	80
D_h	74	76	77
AK liver glycogen	72	74	75

(b) β-Amylolysis

Constant values of reducing power were obtained after incubation of the glycogens for 26 hr. with barley β-amylase. The results for the percentage conversions into maltose were:- Nc 50, N_h 47, Dc 51, D_h 49.

Case IV

The glycogen was kept in solution (6.4 ml.), aliquots being removed for analysis. On complete acid hydrolysis of a sample (ca. 5 mg.) the only sugar detected was glucose.

1. Absorption spectrum of the iodine complex

The following solutions were prepared:-

(A) Glycogen solution (0.27 ml.; 1.4 mg.) was stained with iodine solution (1 ml.) in a total volume of 10 ml.

(B) Glycogen solution (0.27 ml.; 1.4 mg.) was pre-incubated with salivary α-amylase (3 drops; saliva diluted ten-fold) for 54 minutes then stained as above and the volume adjusted to 10 ml.

Solution (A) stained deep red-brown (λmax (H_2O) 440 mμ, E max 0.292); Solution (B) was pale yellow with λmax indistinct (the reading was constant between 420 and 480 mμ) and E max 0.021.

2. α-Amylolysis Limit

Glycogen (12.7 mg.) was incubated at 37° with salivary α-amylase. AK (15.4 mg.) and SK (14.2 mg.) liver glycogens were used as controls and the results are recorded below:-

Table IV (6)

Times of incubation (hr.)	1.5	7	8	9	46
			P_M value		
Case IV glycogen	41	48	48	48	51
AK Liver glycogen	60	76	76	76	80
SK Liver glycogen	26	34	35	35	38

3. Average Chain Length

The average chain length calculated from the P_M value (see page 66) is 7.7 $\pm$ 0.6.

4. β-Amylolysis

On treatment with barley β-amylase (8 mg.) at pH 4.6 the biopsy glycogen (15.8 mg.) gave 3.22 mg. of maltose (19%), 3.87 mg. (23%) and 4.44 mg. (27%) after 5, 20 and 45 hr. respectively.

Discussion

Case II (AK) and Case I (SK)

Case II is characterized by the accumulation of glycogen of normal structure only in the liver and kidney cortex. The absence of excessive glycogen in the muscle or brain is a major difference from cases of generalized glycogenosis (type 2. disease) in which

Table IV (7)

	Liver glycogen (SK)	Liver glycogen (AK)	Kidney glycogen (AK)	Biopsy Sample	Normal Liver (Nc)	Liver glycogen (Dc)	Liver glycogen (Dh)	Amylopectin
Glycogen content of tissues (%)		10.5	4.4	14.6	1.8 (total)		9.4	-
$[\alpha]_D$ water	+201°	+196°	+200°	-	-	-	-	-
Iodine complex λmax (H_2O)	430	460-465	445-450	440	430	440-445	435	535-540
λmax ($AmSO_4$) (mμ)	-	480-490	460-465	-	-	-	-	535-545
Glycogen value	1.61	0.97	1.01	-	0.88	0.92	-	0
α-Amylolysis limit (%)	33	74	76	48	81	80	77	89
β-Amylolysis limit (%) b)	14	46	46	27	50	51	49	61
a)	20	60	59	-	-	-	-	77
Average chain length (glucose residues)	6.3	14.5	14.2	7.6	15.3	14.6	15	23
Exterior chain length	3	9	9	4-5	10	10	10	16-17
Interior chain length	2	4-5	4	2	4	3-4	4	5-6
Molecular weight	2×10^6	7×10^6	7×10^6	-	-	-	-	-
Limiting viscosity no.	11	8	5	-	-	-	-	150

b) before treatment with isoamylase
a) after treatment with isoamylase.

glycogen is stored in large amount in these tissues.

The branching characteristics of the AK glycogens are very similar to those recorded in Table IV (7) for the normal human liver glycogen and also to those of the majority of glycogens in Section 3. The ease of solubility in water, the interaction of the glycogens with iodine and concanavalin-A, the average chain lengths and the P_M values from α-amylolysis differentiate them from the amylopectin-type polysaccharide which accumulates in the type 4. glycogen storage disease, of which there is only one reported case (157). This rules out a deficiency of branching enzyme, amylo-1,4→1,6-transglucosidase.

The exterior chain lengths are much longer than would be expected in a phosphorylase limit-dextrin type of structure; the latter may indicate an absence of amylo-1,6-glucosidase as suggested by Illingworth, Cori and Cori (158).

Having eliminated types 2, 3, 4 and 5 (which is confined to muscle) the conclusion is drawn that the A.K. case is an example of a true von Gierke disease where the accumulation of large quantities of glycogen only in the liver and kidney is the characteristic feature. This indicates that the probable enzymic deficiency is a lack of glucose-6-phosphatase.

Enzymic assay of glucose-6-phosphatase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase was carried out by Dr. P.W. Kent and Mr. A. Calderbank in Oxford. The glucose-6-phosphatase in the diseased liver liberated only 8.3 μg. phosphate/hr./100 mg. tissue

compared to 406 μg. phosphate/hr./100mg. by the same enzyme from a normal liver. The other two enzymes were highly active. Cori and Schulman (159) have reported values of 20 and 17 μg. of inorganic phosphate liberated per hour by glucose-6-phosphatase from diseased liver tissue compared to 362 μg./hour for a normal liver. The virtual absence of glucose-6-phosphatase causes a build up of glucose-6-phosphate as there is no alternative pathway for its breakdown. Glycogenesis continues but as glycogenolysis is incomplete, there occurs an accumulation of glycogen which is continually being synthesized from glucose-1-phosphate by either the phosphorylase or glycogen synthetase mechanisms.

Further studies on the SK liver glycogen characterized by Manners (154), supports the evidence that this has a phosphorylase limit-dextrin type of structure. The high glycogen value, the P_M value and the weak iodine binding power are consistent with very short outer chains and an unusually high percentage of 1,6-linkages. It is not conceivable that this type of structure could have been caused by the method of isolation, which might affect the molecular weight, but certainly would not preferentially degrade the exterior chains in a glycogen-type molecule.

There is evidence that glycogen-storage disease is genetically conditioned and the cases under discussion provide further proof of this as the patients AK (boy, 4 years old) and SK (girl, 12 years old) were siblings. This is the first reported instance of siblings who suffered from different types of glycogen-storage disease. Illingworth and Cori (157) reported the case of a girl (15 months)

with type 1. disease; a sibling died with similar symptoms. Cori and Cori (160) reported a case of male twins ($3\frac{1}{2}$ years) in which the liver glycogen was normal, and enzymic studies, in one case, suggested that they suffered from von Gierke's disease. More recently Hers (161) has provided additional information; two brothers Salvator M. (3 years) and Phillipe M. (5 months) suffered from type 3. disease. Enzymic assay of muscle and liver samples showed a complete absence of amylo-1,6-glucosidase. Two other cases studied by Hers (161) include Maxime D. ($4\frac{1}{2}$ years) whose parents were first cousins and Bruno L. (10 months), who had a cousin with the disease, also suggesting a genetic influence.

In view of the present cases, it seems that a simple recessive trait may have a specific effect on the glucose - glycogen metabolizing system which is able to produce a low level of activity of one of the enzymes, this being chosen at random. Hers (162) has reported a case of type 3. disease in which there was a complete absence of amylo-1,6-glucosidase. A marked decrease in the relative activities of glucose-6-phosphatase, phosphorylase, phosphoglucomutase and cytochrome oxidase in the diseased liver compared to values for normal liver, was also observed. The carbohydrate metabolizing enzyme systems as a whole would seem to have been affected, with a total absence of the debranching system.

Case III

The properties of the diseased liver glycogen are almost identical with that from the normal liver control (Table IV (7)).

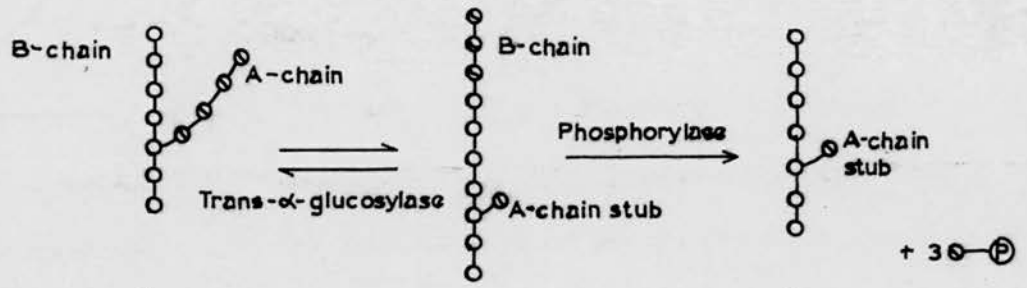
Little difference exists between the polysaccharide isolated by cold and hot water extractions these representing the easier extractable or smaller molecular weight portion and the more firmly bound glycogen, respectively. The large percentage of glycogen in the liver tissue (9.4%) to which glycogen deposition was limited suggests that this case is also a type 1. disease resulting from a reduced glucose-6-phosphatase activity. This has however not yet been substantiated by enzymic assay.

Case IV

The analysis of the glycogen from the biopsy liver sample was limited by the amount of material available, which was part of a very small lobe.

Extraction with 30% potassium hydroxide, was preferred in this isolation, to hot water extraction, because of the good yields of protein-free glycogen which can be obtained. The observed interaction of the polysaccharide with iodine, before and after α -amylase treatment was characteristic of a glycogen. The unusually low P_M value of 48% indicates a higher degree of branching than is normal, corresponding to a calculated average chain length of 7.7 ($\pm$ 0.6) glucose residues. The low β-amylolysis limit of 27% indicates an exterior chain length of 4-5 glucose residues. This value is slightly higher than that for the SK glycogen. These results indicate the presence, in the liver, in high concentration (14.6% tissue weight), of a glycogen of abnormal structure, which resembles a phosphorylase limit-dextrin. In type 3. disease, where there can be little or no debranching of glycogen, it is only the

FIGURE 14



Possible structures for exterior chain stubs in Type 3 glycogen-storage disease. Formula 1 represents a type 3a or 3c structure; formula 2 represents a type 3b structure.

outer chains of the molecules which can be metabolized and it is to be expected that these would vary from an almost normal length, immediately after feeding, to limiting short stubs after fasting. In the present case, the child (Julie G.,; 18 months old) was fasted for some time before the operation, thus it is probable that the glycogen was of the latter type.

This is therefore a type 3. disease, the probable enzymic lesion being a low level of debranching activity.

In view of recent evidence (83) two enzymes may be required to effect the debranching of a phosphorylase limit-dextrin. These are amylo-1,6-glucosidase, and the liver trans- α -glucosylase. Amylo-1,6-glucosidase has no action on β -amylase limit-dextrins but only removes single glucose residues. Absence of the trans- α - glucosylase would leave a phosphorylase limit-dextrin resistant to amylo-1,6-glucosidase action. A lack of either amylo-1,6-glucosidase or the transglucosylase would therefore prevent complete breakdown.

It is suggested that the type 3. disease could be subdivided further; type 3a. due to lack of the transferase, type 3b. due to lack of amylo-1,6-glucosidase and type 3c. due to lack of both. In types 3a. and 3c. the exterior chains would each contain four glucose residues. In type 3b. they would be shorter, averaging 2.5 glucose residues, due to the combined action of the transglucosylase and phosphorylase (Figure 14).

The β-amylolysis limit in structure 2. would be approximately half that of structure 1. assuming similar average chain lengths.

The properties of the biopsy glycogen are slightly different from those of the SK glycogen, in the significantly higher P_M and β -amylolysis limit values. It is suggested that the SK liver glycogen is a type 3b. and that the biopsy is type 3a. or c. These suggestions cannot be confirmed by enzymic assay since no tissue is available.

Summary

1. Four cases of glycogen-storage disease have been examined.

2. Liver and kidney glycogens isolated from one case of glycogen-storage disease (AK) have properties similar to those of normal human liver glycogen ($\overline{CL}$ 14-15) and resemble rabbit liver glycogen in properties such as α-amylolysis limit, β-amylolysis limit before and after isoamylase treatment, and reaction with iodine and concanavalin-A.

3. Glucose-6-phosphatase is at a very low level of activity indicating that AK was an example of a true von Gierke disease.

4. The above case (AK) is a sibling of one (SK) investigated earlier by Manners and found to be suffering from a probable deficiency of amylo-1,6-glucosidase. This is the first reported instance of siblings suffering from different types of the disease.

5. Liver glycogen isolated from a third case has been found on structural analysis to have normal properties which were similar to a normal human liver glycogen. This may also be classed as a type 1. disease, although confirmatory evidence from enzymic assay

is not available.

6. The glycogen isolated from a biopsy liver sample has been found to have an abnormal structure, with short outer chains. This case has been classed as a type 3. disease, involving a decreased activity of the debranching enzyme system.

SECTION V

Part 1

The Action of Z-Enzyme on Starch-Type Polysaccharides

Introduction

Enzymic evidence that amylose contains structural anomalies which prevent complete β-amylolysis was first reported by Peat, Whelan and Pirt (163) who found that crystalline sweet potato β-amylase terminated its action when ca. 70% of the molecule had been converted into maltose. There remained a limit-dextrin which stained blue with iodine, exhibiting maximum absorption at the same wavelength as the original amylose. Subsequently it was shown that amorphous soya-bean β-amylase preparations caused complete hydrolysis of amylose due to the presence of a second enzyme, Z-enzyme (3a). This enzyme was shown to be neither an α-amylase, a phosphatase nor an α-1,6-glucosidase, it was concluded that it was a β-glucosidase since almond emulsin preparations could replace Z-enzyme as the factor required to supplement the β-amylolysis of amylose (3b). It was therefore suggested that the anomalies preventing complete β-amylolysis might be single β-glucose units linked to the main chain (3b). Barker and Bourne (31) suggested that the anomalous β-linkages

need not represent branch-points, but could occur at the non-reducing ends of ca. 30% of the molecules and were formed by a mechanism involving a Walden inversion of the phosphorylase-glucose complex with a growing amylose chain which could then no longer act as an acceptor.

Hopkins and Bird (164) criticised the evidence that Z-enzyme was not an α-amylase on the grounds that amylopectin, the test substrate used by Peat and coworkers, does not give a sufficiently sensitive test for traces of this latter enzyme; α-amylases have a lower affinity for highly branched molecules than for linear substrates. Further experiments were later reported by Peat and Whelan (165) in which they compared the action of salivary α-amylase with that of Z-enzyme preparations. α-Amylase treated amylose had a β-amylolysis limit of 89% compared with 98% for the Z-treated amylose; this appeared to be further proof that Z-enzyme activity was not due to traces of α-amylase.

The β-1,4-glucosidase and laminarinase activities of almond emulsin, were separated from Z-enzyme, by Neufeld and Hassid (4). These workers concluded that, since Z-enzyme was neither a β-1,4- nor a β-1,3-glucosidase, there was no reason to suppose that β-glucosidic linkages were present in amylose.

During studies on the β-amylolysis of amylopectin, Manners and King (166) found an apparent relationship between the enzyme concentration and the β-amylolysis limit. This might indicate the presence of traces of α-amylase, as on prolonged incubation with barley β-amylase containing Z-enzyme, amylopectin gave a

series of apparent β-amylolysis limits which increased with enzyme concentration. More recently, Baba and Kojima (167) showed the presence of small amounts of α-amylase in emulsin preparations.

In the present work, the action of the Z-enzyme present in barley and soya-bean β-amylase preparations has been studied, (a) in the presence of β-amylase and (b) in its absence, either by selective inhibition or isolation of the Z-enzyme.

Methods and Materials

(a) Analytical Methods

The analytical methods used have already been described in Section 2. In the iodine staining experiments with amylopectin β-dextrin, measurements at 540 mμ increased the A.V. to ca.0.5 in the most sensitive region of the Spectrophotometer (Unicam S.F. 500) (c.f. A.V. of ca. 0.1 at 680 mμ).

(b) Enzyme preparations

The properties of the barley β-amylase have already been reported in Section 2. The 'stock' soya-bean β-amylase was prepared by Dr. Khin Maung by the method of Bourne, Macey and Peat (168).

Soya-bean Z-enzyme (α -amylase) was prepared by the following method: Soya-bean flour (73 g.) was extracted with water (5 ml./g.) at pH 5.2 in presence of calcium ions (2 mg./ml.) for 1 hr. at room

temperature. After centrifugation, the supernatant liquid was heat-treated for 15 minutes at 70-71° at pH 6.0, then cooled quickly to room temperature. To the supernatant liquid (200 ml.) after centrifugation, p-chloromercuribenzoic acid (2 ml.; 5×10^{-4}M) was added. After dialysis overnight, against running tap water, the enzyme was finally isolated by freeze drying. Yield: 2 g.

(c) Substrates

(i) Isolation and fractionation of starch

Starch was prepared from Kerr's Pink potatoes (KP) by maceration of the potatoes in ethanol and continual sedimentation from distilled water. The starch (0.5 g./100 ml.) was fractionated in an atmosphere of nitrogen by two methods, (a) aqueous leaching at 98°; (b) with thymol and butanol after complete dispersion of the granular structure for 30-60 minutes at 100°. Amylose was isolated as the N-butanol complex, after three recrystallisations; amylopectin was dialysed against running tap water and freeze dried.

(ii) Amylose β-limit dextrin

Potato amylose (2 g.) was incubated with barley β-amylase (20,000 units; 200 mg.) at pH 3.6 in a total volume of 350 ml. for 48 hr. The β-amylolysis limit was 80%. After dialysis against running tap water the dextrin was isolated as the butanol complex.

(iii) Amylopectin β-dextrin

Amylopectin β-dextrin was prepared from KP amylopectin

(2.6 g.) treated with purified soya-bean β-amylase (1 ml.; 50,000 units) at pH 3.6 for 48 hr. The β-amylolysis limit was 61%. After dialysis the dextrin was isolated by precipitation with alcohol and drying with ether.

Amylopectin β-dextrin was also prepared from a commercial sample of waxy maize starch as above but was isolated by freeze drying.

(iv) Glycogen β-dextrin

Glycogen β-dextrin was prepared by incubation of oyster glycogen and barley β-amylase at pH 4.6. Maltose was removed by dialysis and the dextrin kept in solution.

Results

Action of Barley Z-Enzyme on Amylopectin

(a) Iodine staining measurements

Polysaccharide (25 mg.), barley preparation (52 mg.) 0.2 M-sodium acetate buffer (pH 4.6; 3 ml.), and water to 20 ml. were incubated at 37° for 70 hr. Samples (2 ml.) were removed, heated to inactivate the enzyme and stained with iodine solution (0.2% in 2% potassium iodide solution; 2.5 ml.). The A.V. (540 mμ) of amylopectin β-dextrin decreased from 0.740 to 0.097 and the product showed λmax. 420 mμ and A.V. max. 0.210. No change in iodine staining power of glycogen β-dextrin (λmax. 420 mμ, A.V. max.

0.075) was observed. When only 6.25 mg. of barley preparation were used, the following results were obtained:

λ(mμ):	480	500	520	540	560	580
Initial A.V.	0.545	0.622	0.674	0.680	0.630	0.552
Final A.V.	0.446	0.469	0.472	0.440	0.377	0.319

Effect of enzyme concentration

Amylopectin β-dextrin (5 mg.), 0.2 M-acetate buffer (pH 5.6; 3 ml.) and barley preparation (50, 100, 150 units/mg.) were incubated at 35°, samples being removed at intervals for A.V. determination. The following results were obtained:

Enzyme concentration (units/mg. dextrin)	Decrease (%) in A.V. (540 mμ)	
	6 hr.	23 hr.
50	14	24
100	24	47
150	30	60

Pretreatment with calcium ions and ethylenediaminetetra-acetic acid (EDTA)

Barley preparation (ca. 50 mg.), pretreated with 5 x 10^{-3} M-calcium sulphate or 5 x 10^{-2}M EDTA (pH 4.7) for 30 min. at 37° was incorporated into similar digests to the above. Samples (2 ml.) were removed after 72 hr., giving the following results:

Table V. 1

Fall in A.V. (%)

Barley prep. $\lambda(m\mu)$	Normal	Pretreated with Ca^{++}	Pretreated with EDTA
480	67	80	14
500	75	85	24
520	82	89	27
540	85	91	33
560	86	92	36
580	88	92	38

Fall in A.V. (%)

Original λmax ($m\mu$)	530	530	530
Original A.V.	0.67	0.66	0.68
Final λmax ($m\mu$)	430	430	510
Final A.V.	0.22	0.23	0.49

In a similar experiment with 6.7 mg. of EDTA-treated enzyme, only a slight decrease in iodine staining power was observed:

$\lambda(m\mu)$	480	500	520	540	560	580
Initial A.V.	0.529	0.607	0.675	0.665	0.610	0.531
Final A.V.	0.529	0.573	0.600	0.575	0.517	0.436

(b) Measurement of reducing power

The following digests using (a) acetate buffer pH 4.6, (b) acetate buffer pH 5.6, (c) acetate buffer pH 5.6 (with 10^{-3}M-borate) and (d) B.D.H. Universal buffer pH 5.6 were set up:-

Amylopectin β-dextrin (19.2 mg.; 5 ml.), barley preparation (3,800 units), buffer solution (3 ml.) and water to 20 ml. were incubated at 35°, samples (5 ml.) being removed at intervals for determination of maltose giving the following results:

Table V. 2

	Apparent conversion into maltose (%)		
Age of digest (hr.)	20	70	86
Digest conditions			
(a)	25	33	37
(b)	35	54	55
(c)	35	54	56
(d)	31	49	52

(c) Effect of pH and Various Ions on Activity

Digests were prepared using 0.2 M acetate buffers of (a) pH 3.6, (b) pH 4.8, (c) pH 5.6, (d) pH 6.5, and (e) pH 5.6 containing borate to give a final concentration of 5×10^{-3} M. The A.V. of samples (2 ml.) was measured at intervals. The results are given in the following Table:

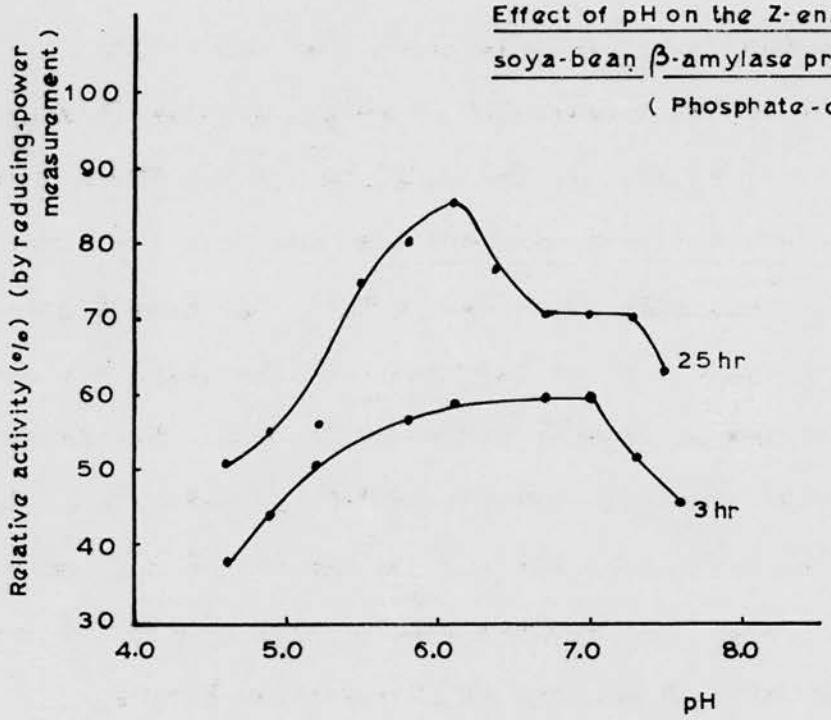
Table V. 3

Fall in A.V. at 540 mμ (%)

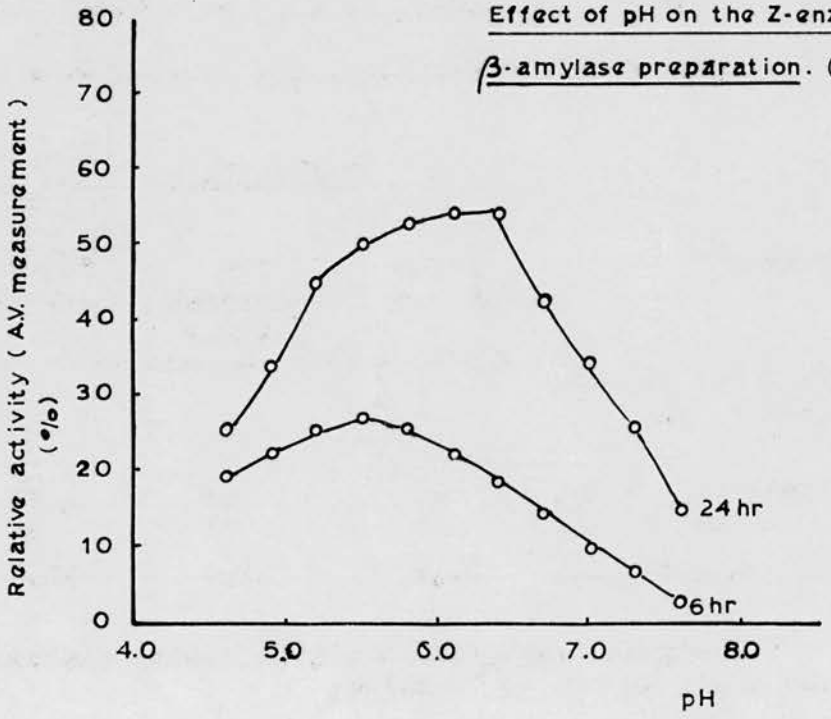
Age of digest (hr.)	1.75	24	48	70	P_M after 70 hr.
Digest conditions					
(a)	0	0	0	0	0
(b)	23	74	83	-	-
(c)	27	87	94	94	45
(d)	-	88	92	93	33
(e)	20	84	90	91	46

Similar results were obtained over the range 460-680 mμ .

FIGURE 15



Effect of pH on the Z-enzyme activity of stock soya-bean β-amylase preparation.
(Phosphate-citrate buffer)



Effect of pH on the Z-enzyme activity of barley β-amylase preparation. (Acetate buffer)

(d) pH Activity Determination

Amylopectin β-dextrin (10 mg.), barley β-amylase (1250 units) 0.2 M-acetate buffer (pH 4.6 - 7.6; 5 ml.) and water to 15 ml. were incubated at 35°. Samples (3 ml.) were removed after 6 and 24 hr. and the A.V. at 540 and 680 mμ were determined. After 6 hr. the maximum decrease at 540 mμ was at pH 5.5; after 24 hr., over the range pH 6.1 - 6.4 (see Figure 15). The change in optimum pH is attributed to the decreased stability of the enzyme in acetate buffer at pH 4-6. The experiment was repeated with phosphate-citrate buffer (pH 5.2 - 7.3; 0.1 M citric acid and 0.2 M-disodium hydrogen phosphate; 3 ml.) in a digest volume of 10 ml. The maximum fall in A.V. (540 mμ) occurred at pH 5.6 after 8.76 hr. and at pH 5.8 after 27 hr.

Digests containing β-dextrin (10 mg.), barley preparation (2,000 units), and buffer (5 ml.) in a total volume of 15 ml. were incubated at 35°. The following results were obtained:

Table V. 4

Buffer	Acetate (pH 5.6)	BDH Universal (pH 5.6)	Phenyl-acetate*	Borate*	Phosphate*
Fall (%) in A.V. (540 mμ):					
after (a) 6 hr.	34	33	34	33	33
(b) 27 hr.	70	69	70	70	70
P_M after 99 hr.	12.6	12.9	13.1	12.5	12.6

* Digests contained 5 ml. of sodium acetate buffer (pH 5.6) and 5 ml. of 2×10^{-2} M-anion.

The function of the calcium ion was examined by incubating enzyme solution (2 ml.) with β-dextrin (10 mg.) and acetate buffer (pH 5.8; 3 ml.) in a total volume of 10 ml. Digest (a) contained barley preparation pre-incubated at 37° and pH 5.8 for 67 hr.; digest (b) contained enzyme solution as above, but also 5×10^{-2} M-calcium acetate; digest (c) was the same as digest (a) except that the enzyme was added to a mixture of β-dextrin and calcium acetate.

Table V. 5

Fall in A.V. at 540 mμ (%)

Incubation (hr.)	4.5	7	24
Digest (a)	13	18	49
Digest (b)	31	45	83
Digest (c)	13	19	51

Action of Barley Z-Enzyme on Amylose and Amylose β-Dextrin

(a) Iodine-staining measurements

The effect of mercuric chloride was examined in digests containing amylose β-dextrin (10 mg.), β-amylase (15 mg.), 0.2 M acetate buffer (pH 5.5; 3 ml.), mercuric chloride solution (1 ml.) and water (6 ml.). Samples (3 ml.) removed after 2.5 hr. gave the following results:

Concentration of $HgCl_2$ (M)	10^{-4}	10^{-5}	10^{-6}	Nil
Decrease (%) in A.V. at 600 mμ:	7	15	46	49

(b) Viscometry

Amylose (70 mg.; 20 ml.; 10^{-5} M with respect to p-chloromercuribenzoate) and β-amylase (25 mg. in 15 ml. of 0.2 M-acetate buffer of pH 4.6; 10^{-5} M with regard to p-chloromercuribenzoate) were incubated in a viscometer at 25° giving the following results:

Time (min.)	15	30	60	97	120	155	205	20	45hr.
$1/\eta_{sp}$	3.13	3.32	3.73	3.94	4.20	4.48	4.65	6.25	6.76

No change in A.V. or reducing power was detected within 24 hr. A control experiment showed that p-chloromercuribenzoate had no effect on the reducing power estimation; in the absence of this inhibitor a marked decrease in A.V. and reducing power was observed together with a fall in viscosity.

Action of Barley and 'Stock' Soya-bean Preparations on Amylopectin

Waxy maize starch (30 mg.), 0.2 M-acetate buffer (pH 4.6; 10 ml.), barley β-amylase or 'stock' soya-bean β-amylase (1300 units), and water to a final volume of 50 ml. were incubated at 37°. Samples (2 ml. for iodine staining; 3 ml. for reducing-power measurements) were removed at intervals giving the following results:

Table V. 6

Time of incubation (hr.)			Time of incubation (hr.)		
	27	49		27	49
	Barley β-amylase			Soya-bean β-amylase	
β-Amylolysis limit (%)	55	54	β-Amylolysis limit (%)	54	55
A.V. (680 mμ)	0.039	0.036	A.V. (680 mμ)	0.037	0.033
A.V. (540 mμ)	0.152	0.128	A.V. (540 mμ)	0.147	0.136

Action of 'Stock' Soya-bean β-Amylase on Amylopectin β-Dextrin

(a) Iodine staining measurements

Waxy maize I β-dextrin (24 mg.; 5 ml.), 0.2 M-acetate buffer (pH 4.6; 3 ml.), 'stock' soya-bean β-amylase (3 ml.; solution prepared by dissolving 50 mg. enzyme in 5 ml. buffer and centrifuging), and water to 25 ml. were incubated at 35°. Samples (2 ml.) were removed after 27 hr. giving the following results:

λ(mμ)	480	500	520	540	560	580	680
Initial A.V.	0.528	0.612	0.687	0.700	0.649	0.574	0.198
Final A.V.	0.034	0.039	0.039	0.038	0.038	0.038	0.017

(b) pH Optimum determination

The effect of pH was examined in digests containing amylopectin β-dextrin (5 mg.; 3 ml.), phosphate-citrate buffer (pH 4.6 - 7.6;

5 ml.), β-amylase solution (2 ml. of centrifuged solution containing 1.3 mg. enzyme/ml.). Samples (3 ml.) removed after 3 hr. gave a maximum decrease in A.V. at pH 7.0. In similar digests using a 1% β-amylase solution (2 ml.) the P_M values of 3 ml. portions were determined after 3 hr. and 25 hr. giving maximum reducing power at pHs 6.7 - 7.0 and pH 6.1 respectively (see Figure 15).

(c) Effect of calcium ions on enzyme stability

'Stock' soya-bean β-amylase (12 mg.) was pre-incubated in acetate buffer pH 6.1 (10 ml.), (a) 5×10^{-3} M with respect to calcium acetate, (b) 5×10^{-3} M with respect to EDTA and (c), alone. Amylopectin β-dextrin (10 mg.; 5 ml.) and water (9 ml.) was incubated with each of the solutions at 35°. Samples were removed for measurement of A.V. and reducing-power giving the following results:

Table V. 7

Decrease in A.V. (%)

Time (hr.)	(a)	(b)	(c)
1	7	-	1
2.5	17	-	3
13	66	6	24
20	76	7	28
42	92	13	50
P_M value after 42 hr.	19	7	14

(d) Effect of mercuric chloride on the enzyme preparation

Digests containing β-dextrin (10 mg.), 0.2 M-acetate

buffer (pH 6.0; 5 ml.), 0.25% β-amylase solution (2 ml.), water, and mercuric chloride (to give final concentrations of 1.5×10^{-5} and 1.5×10^{-6} M). The changes in A.V. are shown in the following Table:

Table V. 8

	A.V.					
λ(mμ):	540	680	540	680	540	680
Time (hr.)	3.25		8		27.5	
Control (no mercuric chloride)	0.228	0.065	0.167	0.040	0.077	0.021
1.5×10^{-6} mercuric chloride	0.235	0.071	0.172	0.046	0.094	0.025
1.5×10^{-5} mercuric chloride	0.256	0.077	0.204	0.055	0.138	0.034
Blank (no enzyme)	0.279	0.104	0.279	0.104	0.279	0.104

The P_M values determined after 8.5 and 27.5 hr. were (a) without mercuric chloride, 15 and 18; (b) 1.5×10^{-6} M, 14 and 18; (c) 1.5×10^{-5} M, 5 and 9.

Experiments with Soya-bean α-Amylase

(a) Action on the β-dextrins of amylose, waxy maize starch and glycogen

The dextrins (ca. 20 mg.) were incubated with enzyme solution (1 ml.; solution before freeze drying), in 0.2 M-acetate buffer pH 5.6 (5 ml.) and water in a total volume of 20 ml. Samples (3 ml.) were removed, deproteinised, and the reducing-power

determined giving the following results:

Table V. 9

β-Dextrins	Time (hr.):	22	47	70	94	116
Amylose		81	-	99.7	103	106
Amylopectin		27	36	38	38	37
Glycogen		1	4.3	6.1	6.3	6.3

(b) Activity determination

The activity of the freeze dried preparation determined by the method of Fischer and Stein (108) was found to be 0.006 units/mg.

(c) Action of soya-bean α-amylase on glycogen

Rabbit muscle III glycogen (50 mg.), 0.2 M-acetate buffer (pH 5.6; 20 ml.) and soya-bean α-amylase (5 ml.; solution before freeze drying) were incubated at 35°. The A.V. and reducing-power were measured at intervals giving the following results:

Table V. 10

Time (hr.)	Fall in A.V. (%) (480 mμ)	P_M (%)
1.25	18	-
2	23	6
2.25	35	9
3.5	39	14
6	52	18
29	-	26

(d) pH Optimum determinations

The optimum pH determined by action on soluble starch in acetate buffer was found to be 7.0 and 6.7 by A.V. measurements after 3 and 7 hr. respectively. In presence of calcium acetate the pH optimum remained at 7.0. The decrease in optimum pH is again ascribed to pH instability which is prevented by addition of stabilizer.

Effect of Complexones on the Action of Z-Enzyme

Digests were prepared by addition of amylopectin β-dextrin (10 mg.; 5 ml.) to barley β-amylase (10 mg./ml.; 2 ml.) pre-incubated with 0.2 M-acetate buffer (pH 7.0; 5 ml.; 2×10^{-2} M with respect to EDTA or diaminocyclohexanetetra-acetic acid (DCHTA; kindly supplied by Dr. T.S. West) for 0.5 hr. at 35°. 'Stock' soya-bean β-amylase and soya-bean α-amylase were incorporated into identical digests. The fall in A.V., on incubation at 35°, was measured giving the following results:

Table V. 11

Time (hr.)	Digest	Enzyme preparation		
		Barley	'Stock' soya	Soya-α
		Fall in A.V. (%)		
3.5	Control	11	73	85
	EDTA	2	0	1
	DCHTA	1	0	0
5.75	Control	20	89	95
	EDTA	13	7	-
	DCHTA	10	7	0

Discussion

Barley Z-Enzyme

The possible degradation of amylopectin and its β-dextrin by the barley preparation was first examined by A.V. measurements at 530-540 mμ , (the wavelength of maximum absorption), with ca. 250 units/mg. Under these conditions, a marked decrease in iodine-staining power was noted. A similar decrease in the iodine-staining power of glycogen β-dextrin was not observed. This decrease in A.V. was increased by pre-incubation of the enzyme with calcium ions and decreased by a similar treatment with EDTA. That these results are due to stabilisation of the enzyme rather than to activation is shown by the data in Table V.5. These results show the presence of weak α-amylolytic activity in the barley preparation.

Only a slight decrease in iodine-staining power at 680 mμ was observed on incubation of the barley preparation (ca. 40 units/mg.) with amylopectin, these being the conditions used by Peat, Pirt and Whelan (3a). This demonstrates the necessity of using varying concentrations of enzyme for the detection of α-amylase.

Various factors affecting the activity of the Z-enzyme were studied. The optimum pH of barley Z-enzyme, in acetate buffer, was 5.5 after a 6 hr. incubation period increasing to pH 6.1-6.4 after 24 hr. This shift, probably due to pH instability of the enzyme, has been frequently observed, and demonstrates the need for careful control of conditions in such digests. The

addition of a stabilizer should minimize such effects. In phosphate-citrate buffer, the optimum pH was 5.6, at which, the activities in acetate and BDH buffer were identical. Addition of borate, which inhibits isoamylase (93) and activates Cladophora amylase (169), to digests in acetate buffer did not alter the activity.

The action of Z-enzyme on amylose β-dextrin was partially inhibited by mercuric chloride (1.5×10^{-6} M and 1.5×10^{-5} M), whilst PCMB (10^{-5} M) which completely inhibits β-amylase, had scarcely any effect (170). PCMB could therefore be used for the selective inhibition of β-amylase.

The above results, supported by reducing-power measurements, were consistent with the view that Z-enzyme was an α-amylase. This was confirmed by examination of the action of the barley preparation, in the presence of PCMB (10^{-5} M), on amylose. As shown by viscometry, slight random hydrolysis occurred, but there was no change in reducing-power or A.V. Caution is therefore required in testing for α-amylase contaminants.

Soya-bean Z-enzyme

'Stock' soya-bean β-amylase was one of the original sources of Z-enzyme. (The activity of such a preparation has therefore been compared with that of barley Z-enzyme). A similar decrease of A.V. was observed with amylopectin β-dextrin at high enzyme concentrations but not at low enzyme concentrations although the preparation did

not contain sufficient α-amylase to affect the β-amylolysis limit of amylopectin.

Behaviour similar to that of the barley Z-enzyme, towards mercuric chloride and calcium ions was also observed. The optimum pH of the 'stock' soya Z-enzyme in phosphate-citrate buffer was 6.7 - 7.0 after a 3 hr. incubation period, this value decreasing to 6.1 after 25 hr. This decrease may again be due to pH instability, in this case the enzyme being more stable on the acid side of the optimum pH, whereas, the barley Z-enzyme was more stable on the alkaline side.

The properties of the 'stock' soya-bean Z-enzyme give the same general idea of weak α-amylolytic activity, but the enzyme-protein is not identical in properties with the barley Z-enzyme, as the pH optima are different.

Soya-bean α-Amylase

An attempt was made to concentrate the α-amylase from soya-beans by extraction in the presence of calcium ions (β-amylase inhibitor, α-amylase stabilizer). The resulting preparation caused rapid production of reducing sugars from amylose and amylopectin β-dextrin but had only limited action on glycogen β-dextrin (6% P_M value). The activity of the preparation, by the method of Fischer and Stein, was 0.006 units/mg. (c.f. 40 units/mg. for the freeze-dried salivary α-amylase preparation described in Section 2). However, higher concentrations of soya-bean

α-amylase caused more extensive degradation of glycogen (52% fall in A.V. and 18% P_M value).

The general failure of soya-bean α-amylase and barley Z-enzyme to attack glycogen β-dextrin is related to the low enzyme concentration and lower affinity of α-amylases for branched molecules. Cunningham and Manners (170) have shown that salivary α-amylase, diluted 50,000 times, also has little action on glycogen β-dextrin but readily attacks amylose and amylopectin β-dextrins. Other observations by the above workers (171) have shown that barley α-amylase, prepared by the same method as the soya-bean α-amylase, causes an increase in the α-amylolysis limit of glycogen. The soya-bean and barley sources had approximately the same α-amylase content (0.17 units/g. flour; calculated from activity determinations on the α-amylase preparations).

The α-amylase in crude almond emulsin preparations, as used by Peat, Thomas and Whelan, had little action on glycogen β-dextrin but on concentration of this enzyme by the method used for the soya-bean α-amylase, limited action was also observed on this polysaccharide (171).

General Conclusions

Barley and 'stock' soya-bean β-amylase preparations contain traces of α-amylase, the concentration being higher in the latter. These have properties generally similar to that of the α-amylase in sweet-almond emulsin (170). An interesting

property of these α-amylases is their behaviour towards complexones such as EDTA and DCHTA which cause a marked lowering in activity; this may find a possible application in the preparation of β-amylase free from α-amylase.

Banks, Greenwood and Jones (172) independently studied the Z-enzyme in various β-amylase preparations by selective inhibition of this latter enzyme and they concluded that Z-enzyme was a dormant form of α-amylase, present before germination. These workers also concluded that the inability of Z-enzyme to attack glycogen differentiated it from normal α-amylases. In contrast, the present study shows that Z-enzyme will, under suitable conditions, attack glycogen and that this property is typical of α-amylases in general.

The fact that the α-amylase contaminants can catalyze a slight random hydrolysis of amylose, explains the complete hydrolysis of this polysaccharide by crude β-amylase preparations. The knowledge that Z-enzyme is an α-amylase does not, however, give any indication of the nature of the structural anomalies in amylose, although the presence of β-glucosidic linkages is now considered to be unlikely; the anomalies are not yet known, but may include one or more of the following possibilities: (a) a linkage other than the α-1,4-type in the chain or existing as a branch point, the latter being more likely, (b) an anomalous residue, with a substituted phosphate group, or oxidised at position 2, 3 or 6; (c) combination of (a) and (b). Glucose residues in the amylose chain can become modified by oxidation (see Section

6) and resistant to β-amylase and phosphorylase; this might occur during the isolation and fractionation of starch, but it is unlikely that such modifications are solely responsible for the incomplete degradation of amylose.

Part 2

Attempted Purification of Soya-bean β-Amylase free from Z-Enzyme

Several attempts have been made to prepare soya-bean β-amylase free from Z-enzyme by the method of Peat, Pirt and Whelan (3a). Although Z-enzyme action can be completely inhibited by preparing digests at pH 3.6 the β-amylase activity is very much reduced; it was therefore desirable to find a method for the preparation of the pure enzyme and the following attempts have been made using our knowledge of the properties of Z-enzyme, these being modifications of the above mentioned method.

Experimental

Soya beans These were purchased from British Drug Houses Ltd. Soya-bean flour was prepared by crushing the beans in a hammer-mill, and this was defatted with several changes of ether solvent until the ether extracts were colourless. Soxhlet extraction with ether was also used for fat removal, this causing no decrease in the β-amylase activity of the flour.

Activity tests: β-amylase activities were measured by the method of Hobson, Whelan and Peat (109).

The method of Smith and Roe (173) was used for α-amylase

activity tests:

Substrate (2 ml.; 0.3% soluble starch solution in 0.2 M-acetate buffer pH 5.6, 0.01 M with respect to calcium acetate) was incubated with enzyme (0.1 ml.) for 0.5 hr. The solution was washed into a 250 ml. flask containing 3 ml. 1.0 N hydrochloric acid in water (ca. 100 ml.) and iodine solution (1 ml.; 0.18% in 1.8% potassium iodide) added. This was compared with a control blank at 620 mμ . The activity was calculated from the expression:

$$\text{Amylase Units/100 ml.} = \frac{\text{A.V. control} - \text{A.V. digest}}{\text{A.V. control}} \times 600$$

Results and Discussion

Preparation of β-amylase (General method)

The following is an example of a typical preparation by the method of Peat, Pirt and Whelan (3a).

Stage

1. Defatted soya-bean flour (515 g.) was shaken with water (5 ml./g.) for 2 hr. in the presence of a few drops of octyl alcohol.
2. The supernatant liquid was adjusted to pH 4.8 (using a pH meter) with 1.0 N-sulphuric acid and centrifuged.
3. The supernatant liquid was heat treated for 30 minutes at 60-61°, then cooled rapidly to room temperature and

centrifuged.

4. Ammonium sulphate (41.8 g./100 ml.) was added slowly, with stirring, to the supernatant solution from stage 3. (1530 ml.), giving a precipitate which was removed by centrifugation.

5. The ammonium sulphate precipitate was dissolved in ca. 130 ml. and dialysed against distilled water for 48 hr. at 0°. The β-amylase solution (202 ml.) was stored under toluene at 0°.

This preparation had an activity of 12,000 units/ml. by the standard activity test. In presence of mercuric chloride (2×10^{-6} M) the activity was 24 units/ml.

The above solution was fractionated with ammonium sulphate (pH 3.7; ammonium sulphate (490 g.), sodium acetate (9.86 g.), glacial acetic acid (29.2 ml.) in 1000 ml. solution). The fraction obtained, between 16.5 and 27% ammonium sulphate, was refractionated twice, the final fraction precipitating between 17.5 and 24% ammonium sulphate being kept. All precipitates were carefully washed with ammonium sulphate solution, the final precipitate being dissolved in 20 ml. ice cold water. The activity was 43,800 units/ml.

Tests for the presence of Z-enzyme

The following results were obtained on incubation of the enzyme (50 units/mg.) with amylose at pHs 3.6, 4.6 and 5.6:

Time (hr.)	pH:	3.6	4.6	5.6
22		74	76	85
48		74	86	96

Enzyme solution (100 units/mg.; pre-incubated at pH 5.6 with 5×10^{-2} M-EDTA) caused 91% conversion into maltose after 48 hr. The above enzyme preparation therefore contained a trace of α-amylase. The preparation was of a suitable purity for use in the determination of the β-amylolysis limit of amylopectin and glycogen, but could only be used for the analysis of amylose at pH 3.6.

A number of soya-bean β-amylase preparations were made by the above method, but all appeared to contain a trace of Z-enzyme.

The Z-enzyme content of soya-beans apparently varies with the sample; the enzyme activities in the soya-bean flour used in this present work (A) were compared with those of a sample obtained from Dr. C.T. Greenwood (B). Flour (10 g.) was extracted with distilled water (50 ml.) by shaking for 2.5 hr. and the supernatant liquid was used for activity tests giving the following results:

Sample	β-Amylase activity (units/ml.)	α-Amylase activity (units/100 ml.)
A	2550	200
B	2560	221

The α-amylase activity was determined in the presence

of ρ-chloromercuribenzoic acid (10^{-5} M) by the method of Smith and Roe (173).

Modification of stages 2 and 3

Since Z-enzyme is stabilized by calcium ions, an enzyme extract (50 ml.) was made 0.01 M with respect to EDTA after stage 1., the pH of the solution being adjusted to 7.0. This solution, diluted to 110 ml. with distilled water, was heated at 60° for 30 minutes, readjusted to pH 4.8 with 1.0 N H_2SO_4 and centrifuged.

	Total activities (units)
Original juice	126,250
Juice + EDTA	114,000
After heat treatment	23,350

An aliquot of the solution, after heat treatment, caused a 22% decrease in the A.V. of amylose β-dextrin in 18 hr. A trace of α-amylase was still present on reaching stage 5.

The above treatment was repeated on a fresh solution at pH 4.8. The decrease in enzyme activity was as follows:-

Time (min.)	Units β-amylase/ml
0	1330
14	960
20	950
30	825
35	780

Aliquots (1 ml.) removed at each of the above times were tested for α-amylase by A.V. measurement in digests containing amylose β-limit dextrin (5 mg.), 0.2 M acetate buffer (pH 5.6; 3 ml.) and calcium ions in a total volume of 25 ml. An 89% decrease in A.V. was observed with the 35 minute sample after a 22 hour incubation period.

Dialysis against EDTA

A preparation carried to stage 5. was dialysed for 72 hr. against 0.05 M-EDTA solution, (adjusted to pH 7.0) at 0°. The α-amylase activity was not reduced. The solution was fractionated with buffered ammonium sulphate and freeze-dried in two separate portions, (a) in 0.2 M citrate buffer pH 4.6; (b) alone. Freeze-drying caused no loss in activity, and this was a convenient method of storing the enzyme.

Potato amylose was completely converted into maltose by the enzyme (a) (140 units/mg.) at pH 5.6 after 42 hr. A 76% conversion into maltose was observed at pH 3.6.

The amount of Z-enzyme present in this preparation was very small indeed since after 23 hr. the enzyme action at pH 5.6 was not complete.

The above treatment was repeated but failed to give β-amylase completely free from Z-enzyme.

Modification of stage 1

Kneen and coworkers (174) found that in the case of malted

barley and wheat, the β-amylase was relatively more unstable at low pHs than the α-amylase when calcium ions were present. These workers selectively removed α-amylase from β-amylase solutions by prolonged dialysis to remove calcium ions followed by a low pH treatment. The removal of α-amylase was found to be more easily effected when the flour extractant used was 5% sodium chloride. This method has been incorporated into stage 1. as follows:-

Soya-bean flour (100 g.) was extracted with 5% sodium chloride (5 ml./g.) for 18 hr. at room temperature. The supernatant solution was dialysed against many changes of distilled water at 0° for 48 hr. The pH was lowered to 3.0 with 1.0 N hydrochloric acid and held at this temperature for 2 hr. Finally the pH was readjusted to 4.8 by the addition of sodium hydroxide, giving a clear, golden yellow, supernatant solution (580 ml.) after centrifugation.

The enzyme solution caused a steady decrease in the viscosity of amylose β-dextrin solution (pH 5.6), showing the presence of α-amylase. Stages 2, 3, 4 and 5 were then carried out under normal conditions, activity tests being done at each stage:

	Total activity	% Initial activity
Before pH treatment	1.088,000	-
After pH treatment	804,000	74
After heat treatment	684,000	63
After ammonium sulphate precipitation	586,800	54
After dialysis	285,000	26
After freeze drying (1 ml. portion)	290,000	No change

Enzyme solution (5,000 units), amylopectin β-dextrin (20 mg.) 0.2 M-acetate buffer and water to 20 ml. were incubated at pH 5.9. The A.V. of aliquots decreased from 0.560 to 0.519 and 0.378 after 18 and 66 hr. respectively, showing the presence of a trace of α-amylase. Amylose was completely hydrolysed to maltose by the preparation.

Removal of calcium by ion exchange

The dialysed enzyme from above (4 ml.) was passed through a Zeo-Karb 225 resin column (sodium form) to remove calcium. Total activities before and after this treatment were 10,000 and 8,750 units respectively. The solution from the column had a total volume of 101 ml.

An aliquot of this solution (5 ml.) was adjusted to pH 3.0 with acetic acid and incubated at pH 3.0 for 1 hour. The total β-amylase activity measured after readjustment to pH 4.6 was 6,900 units. This treatment did not involve a large

loss of β-amylase; however, when it was repeated on a larger scale at 33° a 70% reduction in β-amylase activity occurred. Traces of α-amylase were still detectable, under optimum conditions, even after the above treatment.

pH Treatment of 'purified' soya-bean β-amylase

β-Amylase (10,000 units) was diluted to 15 ml. with water and added to 0.2 M-acetate buffer (pH 3.6; 15 ml.) at 35°. Measurement of β-amylase activity in the normal way and Z-enzyme activity by A.V. decrease in digests with amylopectin β-dextrin gave the following results:-

Time of incubation (min.)	Z-enzyme activity Decrease in A.V. After 23 hr.	β-Amylase activity (% decrease)
0	82	-
16	55	59
30	34	93
60	22	100
120	0	100

After 2 hr. the Z-enzyme activity was not detectable; no β-amylase activity could be detected after 1 hour. However, the 2 hr. sample caused a decrease in viscosity of amylose β-dextrin at pH 5.6 showing the care that must be taken in testing for very low concentrations of α-amylase.

Conclusions

The removal of traces of α-amylase from carbohydrases is a major problem in enzymic studies on α-1,4-glucosans.

Difficulties have been noted with the preparation of amylo-1,6-glucosidase and Q- and R-enzymes by other workers. In the present investigation, the failure to prepare a completely pure β-amylase preparation may have been due to the relatively high initial α-amylase content of the beans used. Fleming (175) using the method on p.143 was able to obtain from a different batch of beans, β-amylase preparations causing only 70-80% hydrolysis of amylose at pH 4.6.

The problem requires further investigation, both with regard to different samples of soya-beans, and the application of other methods of purification. Chromatography on DEAE cellulose and continuous electrophoresis may offer two solutions.

Summary

1. The complete action of ungerminated barley and soya-bean β-amylase preparations on amylose has been shown to be due to the presence of traces of α-amylase, in addition to β-amylase.

2. Both α-amylase contaminants are stabilized by calcium ions and inhibited by mercuric chloride; they have different pH optima and are therefore not identical.

3. The lower activity of the enzymes towards glycogen or glycogen β-dextrin is ascribed partly to concentration effects and partly to a reduced affinity for highly branched substrates.

4. Since Z-enzyme action is α-amylolytic, it is now unlikely that amylose contains β-glucosidic linkages; the nature of the

barriers to β -amylase is not yet known.

5. Various attempts have been made to prepare pure soya-bean β-amylase, free from Z-enzyme. The selective destruction of Z-enzyme was partly accomplished by the removal of calcium ions, using prolonged dialysis or the complexone EDTA.

SECTION VI

The Action of Potato Phosphorylase (P-Enzyme) on ∝-1,4-Glucosans

Introduction

Although the synthetic action of potato phosphorylase (P-enzyme) has been widely studied, relatively little work has been carried out on the reverse process. The limited observations made by other workers can be divided into three sections; studies on (a) amylose, (b) amylopectin and (c) glycogen.

(a) Phosphorolysis of amylose

It has been claimed (176) that amylose is completely degraded by P-enzyme, but Peat and coworkers have suggested that this enzyme, free from Z-enzyme (∝-amylase), would effect only ca. 70% conversion of this polysaccharide into glucose-1-phosphate (3b.).

Baum, Gilbert and Scott (177) have shown that barriers to phosphorolysis can be introduced into amylose by oxygen treatment. Amylose in neutral and alkaline solution was heated to 95° in a stream of oxygen. The phosphorolysis limits were 70-80% by iodine staining measurements, the parent amylose being degraded

ca. 90%. It seems therefore that there are some barriers in amylose to phosphorylase action; whether or not these are artefacts introduced during the fractionation of the parent starch has not been established fully.

(b) Phosphorolysis of Amylopectin

The action of P-enzyme is confined to the exterior chains of amylopectin but there is little agreement on the extent of degradation. The majority of results (3b, 178, 179) indicate that the phosphorolysis limit is at least 10% lower than the β-amylolysis limit and that the P-enzyme limit dextrin is further degraded by β-amylase.

Meyer and coworkers (180) have, however, studied the arsenolysis of amylopectin (in which inorganic phosphate is replaced by arsenate) and on prolonged and repeated enzyme action they found the arsenolysis-limit (as conversion into glucose) to be the same as the β-amylolysis limit (Glucose is the final product of arsenolysis, as glucose-1-arsenate, which may be formed first spontaneously hydrolyses). Meyer reported that the rate of arsenolysis was about ten times slower than phosphorolysis and a thousand times slower than β-amylolysis.

It is unlikely that phosphorolysis and arsenolysis limits would be different but it is uncertain whether P-enzyme can degrade amylopectin to the same extent as β-amylase.

(c) Phosphorolysis of Glycogens

Observations by Swanson (179) indicated that about 20% of rabbit liver glycogen (of unknown chain length) was converted into glucose-1-phosphate by P-enzyme; more recently Fischer (36) has claimed that purified potato phosphorylase has practically no action towards glycogen. Liddle and Manners (181) have measured the glucose-1-phosphate liberated by P-enzyme action on both amylopectin and glycogen, the values being less than the β-amylolysis limits of the same samples. The following Table summarizes some of the results obtained by these workers:

Table VI (1)

Sample	Phosphorolysis (%)	β-Amylolysis (%)
Glycogen:		
Rabit liver I	3	25
Rabbit liver V	11	51
Helix pomatia	7	37
Mytilus edulis VI	9	46
Trichomonas foetus	16	60
Amylopectin:		
Waxy maize starch	45	57

In the present work the phosphorolysis of glycogen and amylopectin-type polysaccharides has been studied. The muscle phosphorylase limits (ϕ - limits) of some of the samples had been

measured by Dr. A.M. Liddle (182). A comparison has been made between the P-enzyme limit, the ϕ-limit, and the degree of β-amylolysis.

In addition, samples of a linear amylose fraction have been treated under different conditions and the phosphorolysis limits determined by estimation of the glucose-1-phosphate liberated, rather than by measurement of the A.V. of the remaining amylose-iodine complex.

Experimental

1. The estimation of inorganic phosphate and glucose-1-phosphate is described in Section 2.

2. Enzyme activity determination. Method: Green and Stumpf (183).

One unit of activity (G. and S. unit) is defined as the amount of enzyme which catalyzes the liberation of 0.1 mg. inorganic phosphate from glucose-1-phosphate in 3 min. in the following digest (A):

0.1 M-Glucose-1-phosphate (1ml.) at 35° was added to 1% Soluble starch (1 ml.), 0.5 M-citrate buffer (pH 6.0; 0.5 ml.) and enzyme solution with water (to 1 ml.) pre-heated to 35°. After incubation for 6 min. enzymic action was stopped by the addition of 5% trichloroacetic acid (5 ml.). A blank digest (B) was prepared as above, leaving addition of the enzyme until after the trichloroacetic acid was added. Coagulated protein was removed

by centrifugation and the inorganic phosphate in aliquot portions (1-3 ml.) was then estimated. The phosphate content of digest B subtracted from that of digest A gave the amount of phosphate liberated by the enzyme.

Activity determinations on crude enzyme preparations were carried out in the presence of 0.2% ammonium molybdate, which inhibits any R-enzyme impurity (184).

3. Preparation of the enzyme

The method of Baum and Gilbert (185) was used for an initial preparation of the enzyme but the purified enzyme solution was too dilute (0.26 units/ml.) to be used for degradative studies. The solution lost its activity rapidly, and could not be stored in the deep-freeze.

The following method, involving ammonium sulphate fractionation of heat-treated potato juice was therefore used. Thanks are due to Dr. H.B. Wright for helpful advice on this part of the work, which is based on an unpublished method of Dr. Dexter French and Dr. Wright.

(a) Potato juice

Thickly peeled potatoes (2.5 Kg.; var. King Edward) were sliced and steeped in sodium hydrosulphite solution (7 g./l.) for 30 min. The slices were washed with distilled water, minced and the juice expressed through fine nylon. The cloudy yellow liquid thus obtained was centrifuged, giving a clear potato-juice,

greenish-yellow in colour (900 ml.; total activity 1710 units).

(b) Heat treatment

The juice was heated rapidly to 50° on a boiling-water bath and maintained at this temperature for 5 min.; solid ammonium sulphate (20 g./100 ml.) was added and the solution was cooled quickly to room temperature. After 70 min. at pH 6.4, the precipitate was removed by centrifugation.

(c) Ammonium sulphate precipitation

The supernatant solution was brought to 35% (w/v) ammonium sulphate concentration by the addition of solid salt; the resulting crude enzyme precipitate was removed by centrifugation and dissolved in 0.05 M-citrate buffer, pH 6.0 (total volume 119 ml.; total activity 990 units). The following stages were carried out in a cold room at 0-2°.

(d) Fractionation with buffered ammonium sulphate solution

Buffered ammonium sulphate solution was prepared by dissolving 50 g. ammonium sulphate in 0.05 M-citrate buffer (pH 6.0) to give a total volume of 100 ml. Addition of ammonium sulphate was made over a period of 15 min., precipitates being removed after a further 15 min. by centrifugation.

Fractionation was carried out to the following ammonium sulphate concentrations, 0-15%, 15-20%, 20-25% and 25-35%.

Fraction	Ammonium sulphate concentration (% w/v)	Activity (G. and S. units/ml.)	Total activity (G. and S. units)
1.	0-15	0.8	13
2.	15-20	25	134
3.	20-25	65	353
4.	25-35	67	502

(e) Purification of the enzyme

Fractions 3 and 4 were combined (855 units; 129 ml.) and refractionated with ammonium sulphate giving the following results:

Fraction	Ammonium sulphate (% w/v)	Activity (G. and S. units)
5.	0-18	42
6.	18-21	214
7.	21-23	250
8.	23-25	107
9.	25-35	171

Fractions 6, 7 and 8 were combined and dissolved in 0.05 M-citrate buffer (pH 6.0; 40 ml.). This solution was added slowly to amylose solution containing 0.1 g. amylose (prepared as described by Gilbert (185)), stirring being continued for 5 min. after addition. The mixture was then centrifuged. The solid was discarded and the supernatant solution was brought to 35% (w/v) ammonium sulphate concentration, the precipitated enzyme being

removed by centrifugation, dissolved in citrate buffer (pH 6.0; 31 ml.), and stored in a deep-freeze. The preparation had an activity of 15.3 units/ml.

Purity of the Enzyme

(a) Test for α-amylase and Q-enzyme

Amylose solution (2 ml.; 2 mg./ml.) 0.05 M-citrate buffer (pH 6.0; 1 ml.) and enzyme solution (1 ml.) were incubated at 35°. Samples (1 ml.) were removed, stained with iodine, and the A.V. determined at 680 mμ giving the following results:

Time (hr.)	A.V.
0	0.250
1.25	0.249
24	0.231

It was concluded that a trace of α-amylase or Q-enzyme was present.

(b) Test for R-enzyme

Waxy maize starch β-dextrin (2 ml.; 4 mg./ml.), 0.05 M-citrate buffer (pH 6.0; 1 ml.) and enzyme solution (1 ml.) were incubated at 35° (A). A similar digest (B) containing ammonium molybdate (final concentration 0.2%) was also set up. The following results at 680 and 540 mμ were obtained:

	A		B	
Time (hr.)	680 mμ	540 mμ	680 mμ	540 mμ
0	0.036	0.103	0.038	0.106
24	0.046	0.129	0.036	0.100

Tests (a) and (b) show that there is slight α-amylase or Q-enzyme and R-enzyme activity, the latter being inhibited by 0.2% ammonium molybdate.

(c) Test for phosphatase

Digests containing sodium β-glycerophosphate (0.1 M) and enzyme solution or buffer were set up. No inorganic phosphate was liberated after 24 hr. showing the absence of phosphatase.

(d) Test for D-enzyme

Pure maltotetraose (20 mg.), citrate buffer (pH 7.0; 2 ml.) and enzyme solution (1 ml.) were incubated at room temperature. After 24 hr. incubation, no other sugars could be detected by ascending paper chromatography (2 ascents). D-Enzyme, the action of which has already been described in Section 1, was therefore absent.

(e) Inhibition of the α-amylase or Q-enzyme contaminant

Digests were prepared in citrate buffer pH 6.0 in the presence of mercuric chloride (final concentrations 3×10^{-4} M, 3×10^{-5} M and 3×10^{-6} M) with amylose β-dextrin as substrate; 0.5 ml. enzyme was used in each digest. Aliquot portions were removed after 16 and 22.5 hr. stained with iodine, and the A.V. measured at 680 mμ.

Molarity of $HgCl_2$:	3×10^{-4}	3×10^{-5}	3×10^{-6}
Time (hr.)		A.V. at 680 mμ	
16	0.360	0.354	0.347
22.5	0.365	0.351	0.341

The A.V. of the control digest without enzyme was 0.356.

The enzyme solution (1.0 ml.) caused no decrease in the specific viscosity of amylose solution in the presence of 3×10^{-5} M-mercuric chloride. It was therefore decided to use this concentration of inhibitor in all digests.

(f) Activity of the enzyme in presence of mercuric chloride

Normal activity tests were set up, the enzyme being pre-incubated for 20 min. with the inhibitor, before the addition of glucose-1-phosphate. The results are given below:

Pre-incubation Molarity of $HgCl_2$ (20 min.)	During incubation	Inhibition (%)
3×10^{-4}	2.1×10^{-4}	19
3×10^{-5}	2.1×10^{-5}	2.5
3×10^{-6}	2.1×10^{-6}	0

It was considered satisfactory therefore to use the enzyme for degradative purposes in the presence of 0.2% ammonium molybdate and 3×10^{-5} M-mercuric chloride.

4. Preparation of linear potato amylose (L)

Potato starch (var. Kerr's Pink) was refluxed with several changes of methanol on a boiling-water bath (186). The starch was dried on a Buchner filter then leached with water at 58° for 8 min. in an atmosphere of nitrogen. The supernatant liquid was poured through a No.3 sintered glass crucible and the amylose then precipitated by the addition of N-butanol. Approximately 600 mg. amylose was obtained from 30 g. of starch. Amylose solutions, for anzyme digests, were prepared by dissolving some of the complex in water and steam distilling off the butanol in an atmosphere of nitrogen.

5. Preparation of maize amylose

Maize starch was leached at 60° for 20 min., the amylose being isolated as above.

Results

Phosphorolysis of Glycogen and Amylopectin

The results of preliminary studies on glycogen and amylopectin are shown in the following Table:

Table VI (2)

Sample	G. and S. units/mg. polysaccharide	Time (hr.):	4	19–25	27	42–48	68–74	90	β-Amylolysis limit (%)
			Phosphorolysis limit (%)						
AK Human liver	0.88		6	6	-	11	16	16	46
Brewer's yeast glycogen	0.74		-	17	19	21	21	-	44
Phytoglycogen A	0.38		-	-	-	19	19	-	50
Floridean starch II	0.48		-	35	-	35	35	-	37
Waxy sorghum starch I	0.61		-	-	-	33	32	-	56
Potato amylopectin (var. Epicure)	0.62		-	43	-	43	43	-	57

Phosphorolysis of Amylose

Aqueous leached amylose L (ca.6.00 mg.) was dissolved in water (60 ml.) and the butanol removed by steam distillation in an atmosphere of nitrogen. The solution was divided into six equal portions which were heated on a boiling-water bath for varying times in the presence of oxygen. A control sample (2) was treated in an atmosphere of nitrogen and sample (6) was the untreated parent amylose. Solutions were buffered to pH values with B.D.H. Universal buffer.

After treatment the amylose was twice reprecipitated as the N-butanol complex. Limiting viscosities were measured in 1.0 M-potassium hydroxide and phosphorolysis was carried out using 0.5 G. and S. units/mg. polysaccharide, the results being calculated from the glucose-1-phosphate liberated after 48 hr.

The results are summarized in the following Table:

Table VI (3)

	pH	Time of treatment (min.)	Atmosphere	$[\eta]$	Phosphorolysis (%)
1.	9.0	30	O_2	237	83
2.	9.0	30	N_2	278	97
3.	7.0	30	O_2	257	88*
4.	Water(5.4)	180	O_2	247	91
5.	9.0	70	O_2	-	92
6.	No treatment			290	98

* This sample had a β-amylolysis limit of 94%, c.f. 99% for the original amylose.

Maize amylose in 0.5 M-sodium hydroxide was heated for 20 min. at 98° in an atmosphere of oxygen. The untreated sample had $[\eta]$ = 145, a β-amylolysis limit of 98%, and a phosphorolysis limit, by A.V. measurement of the amylose-iodine complex, of 97%. The residual amylose, after treatment, had $[\eta]$ = 21, a β-amylolysis limit of 87%, and a phosphorolysis limit of 81% and 73% by A.V. measurement and glucose-1-phosphate estimation respectively.

Discussion

Action of P-Enzyme on Amylopectin

P-enzyme caused fairly rapid degradation of amylopectin the phosphorolysis limit being about 15% lower than the β-amylolysis limit. The results are of the same order as those obtained by Peat and coworkers (3b) who observed 39 and 48% liberation of glucose-1-phosphate for two amylopectin samples using 0.3 G. and S. units/mg. of polysaccharide, c.f. 0.6 units/mg. in the present work.

The outer chain stubs in the P-enzyme limit dextrins of potato, and waxy sorghum amylopectins calculated from the average chain length and average exterior chain length values (see Section 3) are 6 and 8 respectively. The exterior chains are thus appreciably longer than those of ϕ-limit dextrins which are four units long.

Action of P-Enzyme on Glycogen

A very limited action was observed on glycogen, the rate

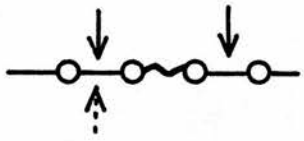
being much slower than that for amylopectin, which indicates a much lower affinity of the enzyme for the former polysaccharide. The ϕ-limit for brewer's yeast glycogen is about 30% (182); there is a marked difference between the action of plant and animal phosphorylases on this polysaccharide. A similar difference exists in the case of waxy sorghum starch which has a ϕ-limit of 40% (182). Other differences between the activities of the two enzymes include the nature of primers and the chemical composition, already mentioned in Section 1. The results with phytoglycogen A show a close relationship to animal glycogens, and with Floridean starch a close relationship to amylopectin. These results are in agreement with those obtained by iodine staining.

It is concluded from the above results, and those obtained by Dr. A.M. Liddle, that P-enzyme is unsuitable for use in structural studies on glycogen and amylopectin, in contrast to β-amylase and muscle phosphorylase which may yield useful information.

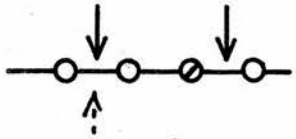
Action of P-enzyme on Amylose

The general results support the conclusions of Gilbert and coworkers (177) in that inadvertent oxidation of amylose during the preparation or fractionation of atarch may result in chemical modification of the constituent glucose residues with the result that the extent of subsequent enzymic degradation may be abnormally low.

The behaviour of P-enzyme and β-amylase on modified amyloses is not identical. This may be due to the fact that phosphorylase action involves the stepwise removal of adjacent glucose residues whilst β-amylolysis involves the hydrolysis of alternative glucosidic linkages. The findings of Peat and Turvey (187) on the presence of nigerose in β-amylolytic digests of Floridean starch suggests that β-amylase may not be completely specific for α-1,4-linked D-glucopyranose residues. The structure shown below, might be resistant to phosphorylase but its sensitivity to β-amylase might depend on whether the anomalous residue was an odd or even number of units from the non-reducing end-group.



Floridean starch



Modified amylose

Key:

—O α-1,4-linked glucose residue

~O α-1,3-linked glucose residue

—⊘ α-1,4-linked modified glucose residue

↓ Linkage susceptible to β-amylase

↑ Linkage susceptible to P-enzyme

The present results are not in exact agreement with those of Gilbert; this may be due to differences in the method of following enzyme action. It is thought that estimation of glucose-1-phosphate is the better method since the presence

of traces of retrograded amylose will interfere with the iodine-staining method. A similar discrepancy in the degree of β-amylolysis of amylose as measured by iodine-staining or the production of maltose has also been observed in this laboratory.

Summary

1. The activity of P-enzyme towards α-1,4-glucosans decreases in the order amylose, amylopectin, glycogen. In the case of branched polysaccharides the limit of P-enzyme action is, in general, considerably less than the ϕ-limit and the β-amylolysis limit.

2. Oxidation of amylose, especially at alkaline pH values, introduces barriers which are resistant to phosphorylase action. These barriers may also be resistant to attack by β-amylase.

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α-1:4-Glucosans

10. GLYCOGEN STRUCTURE AND *RIGOR MORTIS* IN MAMMALIAN MUSCLES*

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Numerous chemical studies have demonstrated that glycogens from various sources may differ in such characteristics as molecular weight, average chain length and in the relative number of D-glucose residues in external and internal chains (Manners, 1957). That constitutional differences in the glycogen molecule have metabolic significance has been indicated by Stetten, Katzen & Stetten (1956), who showed that in the rat and rabbit the larger molecules of muscle glycogen are more reactive, and by Stetten & Stetten (1955), who found that D-glucose residues located peripherally in the chains have a higher rate of turnover than those more centrally situated. Another aspect of the apparent physiological inhomogeneity of mammalian-muscle glycogen has arisen during investigations on post-mortem glycolysis, and on the concomitant onset of *rigor mortis*. In general, provided that pre-slaughter stress has not seriously depleted glycogen reserves, post-mortem glycolysis will continue until pH 5·4–5·5 is reached in the muscles, at which the enzyme systems producing lactic acid are inactivated and some residual glycogen may be found. At a final pH of 5·9, 0·6–1 % of glycogen frequently remains in the psoas and diaphragm muscles of the horse (Lawrie, 1955), and 0·6 % at a final pH of 6·1 in the sternocephalicus muscle of the ox (Howard & Lawrie, 1957). This could signify that the glycogens remaining in such muscles after the completion of *rigor mortis* were either inaccessible or insusceptible to attack during post-mortem glycolysis, or that they might differ in

* Part 9, Eddy, Fleming & Manners (1958).

constitution from the glycogens which predominate in the pre-rigor state. The analytical methods employed in previous papers of this series have therefore been applied to glycogens prepared from some mammalian muscles during the pre- and post-rigor phases.

MATERIALS AND METHODS

Muscles. Samples (0·5–1 kg.) from 7–8-year-old draught horses and 3–4-year-old steers were excised 15–20 min. after death. The following locations were studied: heart (left ventricular wall); diaphragm (junction of pars sternalis with pars costalis); psoas (portion of psoas major at level of sacral vertebrae); longissimus dorsi (l. dorsi; portion posterior to first, second and third lumbar vertebrae); sternocephalicus (entire muscle).

Definition of pre-rigor and post-rigor samples. General features of the time course of *rigor mortis*, and of its associated biochemical changes, in the horse (Lawrie, 1953) and the ox (Howard & Lawrie, 1956, 1957), indicate that muscles from these species, with the exception of horse heart, if sampled when at a pH greater than 6·8 (horse muscles, ox sternocephalicus) or than 6·4 (ox psoas), will then be in the pre-rigor condition provided that they eventually attain a pH of 6·1 (horse muscles, ox sternocephalicus) or 5·7 (ox psoas). Samples of glycogen characteristic of the pre-rigor condition were accordingly prepared from these muscles at a standard time of 1 hr. *post mortem*, when the pH was above the prescribed limit.

Horse heart, however, goes into *rigor mortis* within an hour of death and thus, for practical purposes, yields only post-rigor glycogen, unless special techniques are used.

Samples of glycogen characteristic of the post-rigor phase were prepared from muscle samples held in moist nitrogen at 37° for 5 hr. and thereafter stored for 17 hr. at 0°. The high temperature ensures that conversion of glycogen into lactic acid will occur with the minimum accumulation of hexose phosphate intermediates (Bendall & Davey, 1957); the elapse of 24 hr. from the time of death ensures that the ultimate pH has been attained when the muscle is processed.

pH was determined by glass electrode on samples of muscle, 0·5–1·0 g. being homogenized in 10 ml. of 5 mM-sodium iodoacetate (Bate-Smith & Bendall, 1947).

Preparations of glycogen for analysis. Small pieces cut from the intact muscle were quickly added to hot 30% (w/v) NaOH held in a boiling-water bath (1 g. of muscle to 9 ml. of alkali) and heated until homogeneous. On cooling, ethanol was added to give a final concentration of approximately 50% (v/v). The impure glycogen was centrifuged down after settling overnight and washed with a mixture of 1 vol. of ethanol with 2 vol. of 20% (w/v) NaOH. From this point the procedure followed that described by Somogyi (1957) for the preparation of glycogen free from nitrogen and phosphorus. Each glycogen sample used for structural analysis represented the bulked yields from the muscles of several animals.

Electrophoresis of glycogen. This was kindly examined by Dr D. H. Northcote, using the method of Fuller & Northcote (1956).

Analytical methods

Glucose content. Glycogen was hydrolysed in 2N-sulphuric acid (0·1%) at 100° for 2 hr. and the glucose content of the hydrolysate determined by the Somogyi (1952) reagent. Hydrolysis of glycogen under the conditions devised by Pirt & Whelan (1951) for the analysis of starch (with 1·5N-sulphuric acid) gave values 1–2% lower than the above. The slightly stronger acid is necessitated by the greater proportion of α-1:6-glucosidic linkages. A control experiment showed that no destruction of glucose occurred (reducing-power measurements).

Estimation of glycogen. The procedure of Good, Kramer & Somogyi (1933) was employed.

Enzymic degradation and iodine-staining. These analyses were carried out as described by Liddle & Manners (1957), except that, in the α-amylolysis digests, 3·7 units of enzyme/mg. of polysaccharide was used. P_M denotes apparent percentage conversion into maltose. $E_{max.}$ represents the extinction (absorption value) of the iodine-stained polysaccharide at the $\lambda_{max.}$.

Glycogen value. The interaction of glycogen and concanavalin-A prepared from jack-bean meal was examined under the conditions of Cifonelli, Montgomery & Smith (1956), except that extinctions were measured on a Unicam SP. 500 spectrophotometer. The extinction given under these conditions by 1 mg. of glycogen was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1·00) kindly provided by Professor F. Smith. Amylopectins did not react under these conditions (see Calderbank, Kent, Lorber, Manners & Wright, 1960).

Periodate oxidation. The horse l. dorsi (post-rigor) muscle glycogen (about 250 mg.) was oxidized with a mixture of 20 ml. of 8% (w/v) sodium metaperiodate solution and 80 ml. of 5% (w/v) potassium chloride solution at room temperature, as described by Bell & Manners (1952). A second sample (about 100 mg.) was oxidized with sodium metaperiodate at 2° under the conditions of Manners & Archibald (1957).

The remaining glycogens (about 75 mg.) were oxidized with a suspension of potassium metaperiodate (5 ml. of sodium metaperiodate and 20 ml. of potassium chloride solutions) at room temperature. The oxidation was completed after 9–10 days, and 10 ml. samples were removed for analysis of formic acid after 10 and 12 days. A reagent control was also prepared and analysed.

RESULTS

Glycolytic criteria of horse and ox muscles

Characteristic pre- and post-rigor values for the pH and glycogen concentration in the muscles studied are given in Table 1. There is a considerable concentration of residual (or post-rigor) glycogen in all the muscles except horse heart. In horse l. dorsi and ox psoas, where the final pH is about 5·5, inactivation of glycolytic enzymes presumably accounts for the unattacked glycogen. This reason cannot automatically be adduced for horse psoas and diaphragm and ox sternocephalicus muscles, since the final pH is 5·8–6·0.

Table 1. *Characteristic* pH *values and glycogen contents of muscles investigated*

Values in parenthesis indicate the number of specimens examined. Initial pH and initial glycogen values were obtained 1 hr. *post mortem*.

Muscle	Initial pH	Final pH	Initial glycogen (pre-rigor) (mg./100 g.)	Residual glycogen (post-rigor) (mg./100 g.)
Horse heart	6·14±0·07 (4)	5·86±0·06 (4)	723± 64 (4)	79± 45 (4)
Horse psoas*	6·77±0·04 (6)	5·85±0·06 (6)	1229±190 (6)	606±143 (6)
Horse diaphragm	6·92±0·03 (6)	5·86±0·04 (6)	1883±127 (6)	1184± 43 (6)
Horse l. dorsi	6·87±0·05 (6)	5·45±0·08 (6)	2216±125 (6)	1179±101 (6)
Ox psoas†	6·41±0·07 (12)	5·53±0·07 (12)	772±125 (12)	205± 43 (9)
Ox sternocephalicus	7·11±0·04 (3)	6·01±0·15 (3)	1333±350 (3)	492±110 (3)

* From Lawrie (1955). † From Howard & Lawrie (1956).

Table 2. *Properties of horse- and ox-muscle glycogens*

$E_{max.}$ represents the extinction at $\lambda_{max.}$ after iodine treatment. ECL represents the average length of the exterior chains (no. of glucose residues removed by β-amylase + 2·5).

Sample	Chain length (average)	Glycogen value	$\lambda_{max.}$ (mμ)	$E_{max.}$	P_M	β-Amylolysis limit (%)	ECL
Horse, l. dorsi pre-rigor	17	0·87	480	0·34	83	53	11–12
Horse, l. dorsi post-rigor	17	0·82	490	0·36	78	47	10
Horse, diaphragm pre-rigor	17	0·81	475	0·31	80	53	11–12
Horse, diaphragm post-rigor	17	0·85	480	0·32	80	51	11
Horse, psoas pre-rigor	16–17	—	490	0·32	79	48	10–11
Horse, psoas post-rigor	17	0·76	490	0·35	76	46	10
Horse, heart, post-rigor	16–17	0·83	470	0·29	78	48	10–11
Ox, psoas pre-rigor	16–17	0·86	490	0·34	82	50	11
Ox, psoas post-rigor	15	—	480	0·30	79	50	10
Ox, sternocephalicus pre-rigor	19	0·78	475	0·27	83	51	12
Ox, sternocephalicus post-rigor	15	0·89	485	0·21	79	44	9

Characterization of purified glycogens

Glucose contents. The glycogen samples had glucose contents in the range 96–100 %, and the following analyses are based on these figures.

Iodine staining. The glycogens stained deep red–brown with iodine, and the spectra showed maximum absorption in the range 470–490 mμ. The $\lambda_{max.}$ values in Table 2 represent the mid-point of a wide absorption peak covering 20–30 mμ. Mammalian-liver glycogens under similar conditions show maximum absorption over the range 450–470 mμ ($E_{max.}$ 0·2–0·3), whereas amylopectins have $\lambda_{max.}$ values of 530–540 mμ and $E_{max.} > 0·6$.

Glycogen values. The glycogen values of the muscle glycogens varied between 0·76 and 0·89, indicating a slightly lower degree of branching than usual. There was no appreciable difference between pre- and post-rigor samples.

Average chain length. Potassium metaperiodate oxidation of horse l. dorsi (post-rigor) muscle glycogen (252 mg.) gave formic acid corresponding to chain lengths of 16·9, 16·6 and 16·6 glucose residues after 9, 10 and 11 days. In a duplicate analysis, the production of formic acid from 251 mg. of this glycogen corresponded to an average chain length of 16·6 glucose residues.

Sodium metaperiodate oxidation of the same glycogen (105 mg.) at 2° for 20 days indicated an average chain length of 16·8 glucose residues.

The remaining glycogens were analysed after 10–12 days' oxidation; the sodium hydroxide titres after 10 and 12 days were identical. The production of formic acid indicated average chain length values as follows: Pre-rigor samples: horse l. dorsi, 16·8; horse diaphragm, 16·8; horse psoas, 16·5; ox sternocephalicus, 18·6; ox psoas, 16·5. Post-rigor samples: horse diaphragm, 16·6; horse psoas, 17·1; horse heart, 16·5; ox sternocephalicus, 14·7 and 15·0 in duplicate analyses; ox psoas, 16·5. These values are considered to be accurate to within ±0·5 glucose residue. These results, to the nearest whole number, are summarized in Table 2.

β-Amylolysis limits. Incubation of the glycogens at pH 4·6 and 35° with barley β-amylase resulted in 44–53 % conversion into maltose.

α-Amylolysis limits. On incubation with purified salivary α-amylase at pH 7·0 and 35° for 24 hr., P_M values in the range 76–83 were obtained. The majority of these figures are significantly higher than those observed (approx. 75 %) with mammalian-liver glycogens (average chain length 13–14), and indicate a slightly lower degree of branching.

Electrophoresis. On zone electrophoresis (glass-fibre paper) in 0·1 M-sodium borate buffer, pH 9·3, at 2000 V and 140 mA for 90 min., the glycogens moved 6–8 cm. as one component. The apparent homogeneity of the muscle glycogens may be contrasted with the studies of Lewis & Smith (1957), who found that a variety of glycogens gave two distinct components on electrophoresis in 2 N-sodium hydroxide solution. There were small differences between the mobilities of the pre- and post-rigor samples examined, but these were not in a consistent direction.

The properties of the mammalian-muscle glycogens are summarized in Table 2.

DISCUSSION

Extraction and isolation of glycogen from animal tissues

The conventional solvents for the extraction of glycogen from animal tissues are hot water, cold dilute aqueous (5–10 %) trichloroacetic acid and hot concentrated aqueous (30 %) potassium or sodium hydroxide (Pflüger method). The last-named method has been criticized by many workers (e.g. Meyer & Jeanloz, 1943; Stetten, Katzen & Stetten, 1958; Bryce, Greenwood & Jones, 1958) as causing considerable degradation of the glycogen molecules. There is ample evidence to show that glycogen is degraded by hot dilute alkali (e.g. Greenwood & Manners, 1957; Stetten *et al.* 1958), but the available data indicate that hot concentrated alkali does not cause a progressive reduction in molecular weight. Thus Staudinger (1948) found that treatment of guinea-pig liver and muscle glycogen with 15 or 30 % potassium hydroxide solution at 100° for 1 hr. did not alter the molecular weight (light-scattering measurements). Bryce *et al.* (1958) showed that the sedimentation constant of rabbit-liver glycogen was not progressively reduced after treatment for 3 hr. with hot 30 % potassium hydroxide. Furthermore, Cori & Cori (1958) reported that the distribution of sedimentation constants of glycogen was unaffected after treatment with hot concentrated alkali. This marked difference in the degradative action of dilute and concentrated alkali may be related to the low solubility of oxygen in the latter.

The Pflüger method has therefore been used throughout the present study since it gives a high yield of protein-free glycogen (extraction of muscle tissue with hot water or trichloroacetic acid is extremely inefficient), and does not appear to cause appreciable degradation of the constituent glucopyranosidic linkages.

Previous studies on muscle glycogen

Although the molecular structures of mammalian liver and invertebrate glycogens have been extensively studied (Abdel-Akher & Smith, 1951; Manners, 1957), considerably less attention has been paid to mammalian-muscle glycogens. This may be attributed to the relative difficulty in obtaining adequate samples of tissue, and their low glycogen contents (usually $< 1\%$). The presence of glycogen in muscle tissue was first noted by Sanson (1857), but some 80 years elapsed before the first chemical investigations were reported (Young, 1937; Bell, 1937). These indicated that rabbit-muscle and liver glycogens had similar properties, and that a sample of horse-muscle glycogen (pooled tissues) had an average chain length of 11–12 by methylation. A summary of the results of other end-group assays of muscle glycogens is given in Table 3. These differ markedly from those reported in Table 2. Furthermore, the β-amylolysis limits ($42 \pm 4\%$) are significantly lower than those of our present analyses.

Molecular structure of pre- and post-rigor muscle glycogens

Although glycogens have been isolated with average chain length values ranging from 6 to 18, the majority of samples have values of 12 ± 2 (Manners, 1957). The most noticeable feature of the mammalian-muscle samples as a whole (and, in particular, of the sternocephalicus pre-rigor glycogen) is

Table 3. *Properties of some mammalian-muscle glycogens*

Methods of assay are indicated by: (m) methylation; (p) periodate oxidation; (e) enzymic.

Sample	Chain length (average)	β-Amylolysis limit (%)	References
Horse	11–12 (m, p)	42	Bell (1937); Bell & Manners (1952)
Human	11 (p)	—	Halsall, Hirst & Jones (1947)
	12 (p)	41	Bell & Manners (1952)
	11 (p)	40	Liddle & Manners (1957)
Rabbit	13 (p)	—	Halsall *et al.* (1947)
	11–13 (m, p)	45	Bell (1948*a*); Bell & Manners (1952)
	11 (p)	39	Liddle & Manners (1957)
	15 (e)	—	Illingworth, Larner & Cori (1952)
	13 (p)	46	Manners & Wright (unpublished work)

therefore the significantly lower degree of branching, as determined by potassium periodate oxidation. This method is well established in carbohydrate chemistry for the analysis of non-reducing polysaccharides, and the possibility of any inherent error is reduced by the close agreement reported by other workers between methylation, enzymic and periodate-oxidation analyses of the same samples.

The potassium periodate-oxidation results in Table 2 are supported by the glycogen and P_M values.

The interaction of concanavalin-A and glycogen was first examined in detail by Cifonelli *et al.* (1956), who noted that the glycogen values of various glycogens were increased by β-amylolysis. They concluded that the reaction mainly involved the interior chains. However, it seems probable that other factors are also significant since measurement of the glycogen values of various human glycogens (from cases of glycogen-storage disease) have indicated an approximate relationship with the degree of branching (Calderbank *et al.* 1960). The glycogen values of the horse-muscle glycogens are significantly lower than those of 12–14 unit glycogens and are therefore consistent with the lower degree of branching and the relatively longer exterior chains.

The extent of degradation by α-amylase may, to a first approximation, be related to the degree of branching in the polysaccharide, since the interchain linkages and certain adjacent α-1:4-glucosidic linkages are resistant to enzyme action (Whelan & Bines, 1955). The observed trend of P_M values for the muscle glycogens is fully in accord with the significantly lower degree of branching, as the values are intermediate between those of a normal glycogen (average chain length 14, P_M 75) and an amylopectin (average chain length 22, P_M 89).

The iodine-staining power of mammalian-muscle glycogen is appreciably greater than that of liver or invertebrate glycogens (Manners, 1957). However, this feature of muscle glycogens, which has been noted by other workers (Young, 1937; Bell, 1948*b*), is not apparently directly related to the molecular structure. The iodine-staining data in Table 2 serve to characterize the muscle polysaccharides as 'glycogens' rather than 'amylopectins'. For example, the amylopectin component of malted barley starch has an average chain length of only 18 (Aspinall, Hirst & McArthur, 1955) but has $\lambda_{max.}$ 540 mμ and $E_{max.}$ 0·77 (unpublished data).

The results recorded in Tables 1 and 2 show that the glycogens remaining after the development of *rigor mortis* in horse muscles, and in ox psoas, do not differ significantly in molecular structure from the initial pre-rigor polysaccharides. On the other hand, in ox sternocephalicus muscle there is a significant shortening of the outer chains, from 12 to 9 glucose residues, during post-mortem glycolysis.

Biochemical and physiological implications

The biochemically significant quantity of glycogen which remains unattacked by the glycolysis system at a final pH of 6·0 in ox-sternocephalicus muscle appears to represent a molecular species having a shorter average external chain length than that initially present. In this instance, the presence of residual glycogen at a high final pH may be rationalized at the chemical level. Although in horse psoas and diaphragm residual glycogen at a high final pH is found, this does not apparently differ from the pre-rigor glycogens in these muscles. The pre- and post-rigor glycogens in these muscles need not be physiologically equivalent, however, since fractionation of a given glycogen sample can yield species of different molecular weight but similar branching characteristics (Stetten & Stetten, 1958). This may have physiological importance as glycogen of high molecular weight appears to be preferentially degraded *in vivo* and *in vitro* by muscle phosphorylase (Stetten *et al.* 1958; Larner, Ray & Crandall, 1956) and to exchange its glucose units more vigorously with the environment than that of low molecular weight (Stetten *et al.* 1956). In general, post-rigor glycogens would thus be expected to have a low molecular weight and a more compact molecular-weight distribution. If this is so, there would seem to be no relationship between the degree of branching and molecular size in the horse-muscle glycogens examined.

Dr W. A. J. Bryce has kindly determined the sedimentation constants of the l. dorsi muscle glycogens; the polysaccharides were polymolecular, values of 96 and 80×10^{-13} c.g.s. unit being obtained for the major components of the pre- and post-rigor samples, corresponding to molecular weights of 4·4 and $3{\cdot}7 \times 10^6$ [assuming a diffusion constant of $1{\cdot}50 \times 10^{-7}$ (Bell, Gutfreund, Cecil & Ogston, 1948)]. These results are qualitatively in agreement with the above suggestion.

In view of the similar average chain length values of the pre- and post-rigor glycogens from horse muscle and ox psoas, and assuming that the major pathway of glycogen breakdown involves phosphorylase, it is clear that the relative activity of phosphorylase and amylo-1:6-glucosidase is largely unaltered during post-mortem glycolysis. This may be contrasted with other metabolic conditions, e.g. glycogenesis, in which an alteration in activity is manifested by the presence of glycogen with relatively short exterior chains and a higher degree of branching (Manners, 1957). On the other hand, with ox-sternocephalicus muscle, the significant shortening of the outer chains from 12 to 9 glucose residues may indicate either a partial inactivation

of the debranching enzyme, or *in vivo* heterogeneity with respect to branching characteristics.

The concentration of glycogen in muscle has hitherto been recognized as an aspect of functional specialization, since it tends to be high in so-called white muscles, which derive energy for short bursts of activity by anaerobic glycolysis, and low in so-called red muscles, which are equipped for sustained energy production by respiration. That this relatively simple concept might be inadequate was indicated by the work of Bloom, Lewis, Schumpert & Shen (1950) and Kits van Heyningen & Kemp (1955). It now appears that distinct molecular species of glycogen, exhibiting different susceptibilities to glycogen breakdown, may occur in a given muscle. This presumably reflects some further aspect of functional specialization.

SUMMARY

1. The molecular structure of glycogen isolated by the Pflüger method from some mammalian muscles during the pre- and post-phases of *rigor mortis* has been examined by chemical and enzymic methods.

2. Glycogen from pre- and post-rigor horse-muscle tissues has an average chain length of approximately 17 glucose residues. This indicates a significantly lower degree of branching than is present in other samples of mammalian and invertebrate glycogen.

3. The glycogen isolated from ox-sternocephalicus muscle after the onset of *rigor mortis* has significantly shorter exterior chains than that isolated from the pre-rigor muscle. This could indicate either partial inactivation of the enzyme system catalysing the glycogen $\rightleftharpoons$ glucose 1-phosphate interconversion or a true heterogeneity of the glycogen in this muscle *in vivo*.

4. The chemical and physiological significance of these observations is discussed.

5. The possibility of the degradation of glycogen during alkaline extraction is considered.

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Biochemical Investigation of a Case of Glycogen-Storage Disease (von Gierke's Disease)

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Glycogen-storage disease is characterized by the deposition of unusually large quantities of glycogen, particularly in the liver and kidneys. In a survey of a number of cases, Cori (1952–53) has recognized the existence of four types of the disease. In type 1 (also known as von Gierke's disease), the physical properties and the chemical structure of the glycogen appear to be normal and the biochemical defect lies in a striking deficiency of glucose 6-phosphatase both in the liver and kidney. Type 2 is shown by the generalized deposition of glycogen in many tissues, notably in heart and skeletal muscle. The glycogen has a normal structure, glucose 6-phosphatase is present and the biochemical abnormality has not so far been defined. In types 3 and 4, of which few cases are known, the glycogen accumulated in the liver and kidney has an unusual structure; in type 3 it has very short outer chains and in type 4 the glycogen resembles amylopectin in having relatively long inner and outer branches.

The present studies are concerned with the investigation of a fatal case of the disease in which the molecular structure of the liver and kidney glycogen and the enzymic activity of the liver have been examined. The patient (A.K.) was a sibling of one (S.K.) investigated earlier (Manners, 1954), which corresponded to type 3 of Cori's classification. This

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liver glycogen (S.K.) had an average chain length of only six glucose residues (i.e. a degree of branching twice that of a normal glycogen), the molecular structure resembling that of a phosphorylase-limit dextrin. In this case, a deficiency in amylo-1:6-glucosidase was probable. Some additional properties of the S.K. glycogen are also reported.

METHODS AND MATERIALS

Normal liver. This was from a 2-year-old child who died after a short illness. The liver was removed 21 hr. after death and stored at −18°.

Diseased liver. Tissues were removed from the refrigerated body of a 4-year-old boy (A.K.) 50 hr. after death and were stored at −18°. A portion of the tissue was dropped into boiling water and heated for 10 min. Glycogen was later extracted from this boiled tissue (see Results section).

Dipotassium D-*glucose* 6-*phosphate.* This was prepared from the barium salt (104 mg.; C. F. Boehringer und Söhne, Mannheim, Germany) in water (1 ml.) by addition of solid K_2SO_4 (38 mg.). The solution was centrifuged and the supernatant fluid was made up to 2 ml. with 0·2 M-potassium citrate buffer, pH 6·5.

Dipotassium α-D-*glucose* 1-*phosphate.* This was obtained by phosphorolysis of starch. A solution (0·02 M) made in 6 mM-$MgSO_4$ was used.

Triphosphopyridine nucleotide. This was kindly given by Dr H. L. Kornberg.

Analytical methods

Glucose content. The glycogens, 0·1% concentration, were hydrolysed in 2 N-H_2SO_4 at 100° for 2 hr. and the reducing sugar was determined with the Somogyi (1952) reagent.

Paper chromatography. Descending chromatograms were carried out at room temperature on Whatman no. 1 paper with ethyl acetate–pyridine–water (10:4:3, by vol.) as solvent, and aniline oxalate as development reagent.

Absorption spectra of iodine complexes. The conditions of Peat, Whelan, Hobson & Thomas (1954) were used.

Periodate oxidation. The glycogens were oxidized at room temperature with potassium metaperiodate (Bell & Manners, 1952). The liver glycogen was also oxidized with a limited excess of sodium metaperiodate under the conditions of Manners & Archibald (1957).

Glycogen value. A solution of concanavalin A was prepared from a 0·9% NaCl extract of jack-bean meal (Cifonelli & Smith, 1955). For the determination of glycogen values, 9 ml. of the above solution was added to 1 ml. of aqueous glycogen solution containing 0·1–1·0 mg. of polysaccharide, and the extinction measured after 10 min. at 420 mμ with a Unicam SP. 500 spectrophotometer. The extinction given by 1 mg. of glycogen under these conditions was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1·00) kindly provided by Professor F. Smith (see Cifonelli, Montgomery & Smith, 1956).

Viscosity measurements. The viscosity of glycogen solutions of various concentrations was measured at 25° in a modified Ubbelohde viscometer. The limiting viscosity number $[\eta]$ was obtained by linear extrapolation of η sp./C to zero concentration (C as g./ml.).

Phosphate estimations. These were performed by the method of Berenblum & Chain (1938).

Enzymic degradation: α- *and* β-*amylolysis.* The enzyme preparations and conditions of hydrolysis were those described by Liddle & Manners (1957). α-Amylolysis results are given as P_M values, i.e. apparent percentage conversion into maltose.

Isoamylase. This enzyme (cf. Manners & Khin Maung, 1955) was extracted from brewer's yeast by Miss Z. H. Gunja.

Enzymic assays

Glucose 6-*phosphatase estimation.* Homogenates were prepared by grinding 1 g. of abnormal liver in a chilled mortar. The homogenate was stirred with ice-cold glass-distilled water, filtered through muslin and the filtrate made up to 3·5 ml. A homogenate of normal liver (800 mg.) was prepared similarly except that the final volume of the supernatant fluid was 1·8 ml. Incubations were carried out at 30° for 15, 30 or 60 min., when the reaction was terminated by the addition of 1 ml. of trichloroacetic acid (10%, w/v). Water (1 ml.) was then added and the mixture was centrifuged (445 *g* for 5 min.). A sample (1 ml.) of the supernatant liquid was withdrawn for determination of phosphate.

Phosphoglucomutase estimation. Abnormal liver (1 g.) was homogenized with glass-distilled water (3 ml.) in a Potter glass homogenizer. The product was centrifuged (30 min. at 10 000 *g*) and the supernatant liquid was used for enzyme assay immediately, and after standing for 24 hr. at 2° and being diluted 10-fold, 100-fold and 500-fold with the cysteine buffer. Glucose 1-phosphate was incubated at 30° with the enzyme preparation (0·1 ml.), in the presence of 0·05 M-cysteine brought to pH 7·5 by addition of 0·1 N-NaOH, for 5 or 10 min. The reaction was terminated by the addition of 5 N-H_2SO_4 (1 ml.) and the solution was diluted with water to a final volume of 5 ml. The resulting solution was heated on a boiling-water bath for 3 min., cooled, centrifuged (445 *g*, 3 min.) and 1·5 ml. of the supernatant was withdrawn for determination of phosphate.

Glucose 6-*phosphate dehydrogenase estimation.* Abnormal liver (1 g.) was homogenized with water (4·5 ml.) in an all-glass apparatus at 0° and centrifuged (3500 rev./min.) for 20 min. The supernatant liquid was used for enzyme assay. In parallel experiments, normal liver (850 mg.) was homogenized in water (2·6 ml.) in the same way. Incubation mixtures were prepared containing 0·25 M-glycylglycine (pH 7·6, 3·5 ml.); 0·1 M-$MgCl_2$ (3·5 ml.); water (5·6 ml.); enzyme preparation (0·7 ml.); triphosphopyridine nucleotide (TPN; 3·5 ml. containing 1·4 mg.). For the 'zero time' reading (i.e. immediately after addition of the enzyme), 2·4 ml. of the mixture was mixed with ethanol (2·4 ml.) and 10% (w/v) Na_2SO_4 (0·1 ml.). After 15 min. the solution was centrifuged (2000 rev./min.) and the extinction of the supernatant was measured at 340 mμ. The reaction commenced when 0·05 M-dipotassium glucose 6-phosphate (0·6 ml.) was added to the remaining incubation mixture at 20°. Samples (2·4 ml.) were withdrawn and investigated at various intervals in the manner described. Spectral measurements were made in comparison with an 'enzyme blank' identical with 'zero time' incubation mixture save that the TPN was substituted by an equivalent volume of water.

RESULTS

Isolation and purification of A.K. glycogen

The boiled liver tissue (243 g.) was ground with sand and extracted three times with boiling water in an atmosphere of nitrogen. The combined extracts were cooled to 0° and deproteinized by the addition of 0·1 vol. of 40 % (w/v) trichloroacetic acid. After removal of protein, the glycogen was precipitated with ethanol, purified by five further precipitations and dried with ether. Yield, 18·9 g. (equivalent to 7·8 % yield from the tissue).

Glycogen was similarly extracted from boiled kidney tissue (78 g.). Yield 3·4 g. (i.e. 4·4 % yield from the tissue). Extraction of the kidney residue with 30 % (w/v) KOH at 100° did not yield any additional glycogen.

Boiled brain and muscle tissue were extracted successively with water and 30 % KOH at 100°. No glycogen could be isolated.

Characterization of purified A.K. glycogens

The liver glycogen had $[\alpha]_D + 196°$ (*c*, 0·2 in water), and on acid hydrolysis gave glucose (96 %) and no other sugar. The aqueous solution stained deep red–brown with iodine ($\lambda_{max.}$ 460–465 mμ; $E_{max.}$ 0·21).

The kidney glycogen had the following properties: $[\alpha]_D + 200°$ (*c*, 0·2 in water); glucose content 98 %; iodine complex, $\lambda_{max.}$ 445–450 mμ, $E_{max.}$ 0·19.

Periodate oxidation. Liver glycogen (262·2 mg.) gave 5·06, 5·11 and 5·11 mg. of formic acid after potassium metaperiodate oxidation for 9, 14 and 16 days. The final figure corresponds to an average chain length of 14·6 glucose residues. In a duplicate experiment, 261·0 mg. of liver glycogen gave 5·17 mg. of formic acid on complete oxidation, equivalent to average chain length 14·5.

Kidney glycogen (253·7 mg.) was similarly oxidized; the final production of formic acid, after 17 days, was 5·06 mg., equivalent to average chain length 14·3. In a repeat oxidation, 253·7 mg. of glycogen gave 5·07 mg. of formic acid, i.e. average chain length 14·1.

Liver glycogen (100·3 mg.) in water (22 ml.) was then oxidized at 2° with 3 ml. of 8 % (w/v) sodium metaperiodate. The final production of formic acid, after 20 days' oxidation, was 2·0 mg., corresponding to an average chain length 14·4 glucose residues.

Glycogen values. The measurement of the extinction of 1 mg. of A.K. glycogen in the presence of concanavalin-A gave glycogen values: liver glycogen, 0·97; kidney glycogen, 1·01. Samples of amylopectin isolated from potato starch (var. Kerr's Pink) and the starch from the freshwater alga *Dunaliella bioculata* (Eddy, Fleming & Manners, 1958) did not react with concanavalin-A.

Limiting viscosity number. In 0·1 M-NaCl solution, the liver and kidney A.K. glycogens had $[\eta]$ 8·3 and 5·3 respectively. These figures are about one-twentieth of those for amylopectin.

Enzymic degradation

α-Amylolysis. The results of a comparison of the action at 37° of purified salivary α-amylase on the human glycogens, and on control samples of rabbit-liver glycogen (average chain length 13·8) and potato amylopectin (average chain length 22·0) are shown in Table 1.

β-Amylolysis. On treatment with barley β-amylase at pH 4·6 and 37°, the liver and kidney glycogens both had a β-amylolysis limit of 46 %. Under similar conditions, the rabbit-liver glycogen and potato amylopectin gave 53 and 61 % conversion into maltose.

β-Amylolysis after pretreatment with isoamylase. The glycogens (26–28 mg.) were incubated with isoamylase (50 mg.) at pH 5·9 for 24 hr. at 20° in a total volume of 10 ml. The enzyme was inactivated by heating; 5 ml. of 0·2 M-acetate buffer, pH 4·6, β-amylase (2600 units) and water to 25 ml. were then added. After 24 hr. at 37° the β-amylolysis limits were: liver glycogen 60 %, kidney glycogen 59 %. Potato amylopectin on similar treatment had a β-amylolysis limit of 77 %. The outermost branch points in these polysaccharides are therefore of the α-1:6-glucosidic type.

Further investigation of S.K. glycogen

This glycogen, the structure of which has already been discussed by Manners (1954), gave a pale-yellow–brown stain with iodine and the solution did not show an absorption peak in the region 440–480 mμ; at 430 mμ the extinction was only 0·05. The glycogen had glycogen value 1·61, $[\eta]$ 10·6 in 0·1 M-NaCl and, on α-amylolysis (see Table 1), the P_M values were 24, 27 and 33 respectively. After treatment with isoamylase, the β-amylolysis limit was 20 %.

Table 1. *Action of salivary α-amylase on polysaccharides*

The polysaccharides (0·5 mg./ml.) were incubated at 37° with freeze-dried α-amylase (2·3 units/ml.) in the presence of 0·05 % NaCl in a total volume of 100 ml. Samples (5 ml.) were analysed, at intervals, for reducing sugar (as maltose).

	P_M		
Time of incubation (hr.) ...	2·5	5	24
Liver glycogen (A.K.)	62	66	74
Kidney glycogen (A.K.)	63	67	76
Rabbit-liver glycogen	65	69	75
Potato amylopectin	74	79	89

Determination of liver-enzyme activities

A survey of the activity of four enzymes, namely glucose 6-phosphatase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase, was carried out on homogenates of the diseased (A.K.) liver. Results of six experiments showed that with incubation times between 15 and 30 min. the tissue hydrolysed glucose 6-phosphate at a rate of 1·81–2·72 μmoles/hr./g. of frozen tissue. Under identical conditions, enzyme preparations of normal liver hydrolysed the substrate much more quickly, at a rate of 129·8–130·0 μmoles/hr./g. of frozen tissue.

Table 2. *A comparison of the properties of glycogens from glycogen-storage disease*

Property	Kidney glycogen (A.K.)	Liver glycogen (A.K.)	Liver glycogen (S.K.)
$[\alpha]_D$ (water)	+200°	+196°	+201°
Iodine complex $\lambda_{max.}$ (mμ)	445–450	460–465	—
Glycogen value	1·01	0·97	1·61
Average chain length (glucose residues)	14·2	14·5	6·1
α-Amylolysis limit (%)	76	74	33
β-Amylolysis limit (%) (*b*)*	46	46	14
β-Amylolysis limit (%) (*a*)*	59	60	20
Molecular weight (see Addendum)	7×10^6	7×10^6	2×10^6
Limiting viscosity number	5	8	11

* (*b*) Before and (*a*) after treatment with isoamylase.

Table 3. *Glucose 6-phosphatase activity of abnormal- and normal-liver homogenate*

In these experiments the substrate was 0·1 M-dipotassium glucose 6-phosphate (0·1 ml.) in 0·1 M-potassium citrate buffer (pH 6·5). The enzyme preparations are as described in the Methods and Materials section. Final volume, 0·5 ml. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate found (μg.)	Phosphate liberated/hr./g. of frozen tissue (μmoles)
Abnormal liver (A.K.)			
Buffer (0·2 ml.); enzyme (0·2); water (0·1 ml.)	60	32·5	—
Buffer (0·2 ml.); substrate; water (0·2 ml.)	60	3·75	—
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	0*	37·2	—
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	15	38·0 (38·4)	1·81 (2·72)
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	30	39·5 (39·5)	2·60 (2·60)
Buffer (0·2 ml.); substrate; enzyme (0·2 ml.)	60	42·0 (42·0)	2·72 (2·72)
Normal liver			
Buffer (0·3 ml.); enzyme (0·1 ml.); water (0·1 ml.)	15	40·0	—
Buffer (0·3 ml.); substrate; water (0·1 ml.)	15	12·25	—
Buffer (0·3 ml.); substrate; enzyme (0·1 ml.)	15	97·0	129·8
Buffer (0·3 ml.); enzyme (0·1 ml.); water (0·1 ml.)	30	38·9	—
Buffer (0·3 ml.); substrate; water (0·1 ml.)	30	13·7	—
Buffer (0·3 ml.); substrate; enzyme (0·1 ml.)	30	148·8	130·0

* Zero-time control; trichloroacetic acid was added before the enzyme.

Table 4. *Phosphoglucomutase activity of abnormal liver*

The reaction mixtures were made with 0·02 M-dipotassium α-glucose 1-phosphate (0·1 ml.) as substrate. The buffer mixture contained 0·05 M-cysteine (0·2 ml., pH 7·5) and 6 mM-$MgSO_4$ (0·1 ml.). The enzyme preparation is as described in the Methods and Materials section. Final volume, 0·4 ml.; incubated at 30°. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate liberated (μg.)	Hydrolysable phosphate used (μmoles)
Enzyme; buffer	10	26·65	—
Enzyme; buffer; substrate*	10	93·0	—
Substrate; buffer; water (0·1 ml.)	10	67·7	—
Enzyme; substrate; buffer	5	31·3 (29·3)	2·04 (2·10)
Enzyme; substrate; buffer	10	31·3 (30·0)	2·04 (2·08)

* Zero-time control; sulphuric acid was added before the enzyme.

Table 5. *Phosphoglucomutase activity of aged and diluted enzyme preparations of abnormal liver*

Tubes contained 0·05 M-cysteine (0·2 ml.) and enzyme (0·1 ml., appropriate preparations are described in the Methods and Materials section). In some cases, 6 mM-$MgSO_4$ (0·1 ml.) was also added. Final volume, 0·4 ml.; incubated at 30° for 5 min.

Additions	Hydrolysable P remaining (μg.)	Glucose 1-phosphate used (μmoles/hr./g. of frozen tissue)
Enzyme (1:10); $MgSO_4$	7·37	—
Enzyme (1:100); $MgSO_4$	4·37	—
Glucose 1-phosphate; water (0·1 ml.)	68·2	—
Glucose 1-phosphate; enzyme (1:10)	40·7	5088
Glucose 1-phosphate; enzyme (1:100)	63·0	1536
Glucose 1-phosphate; enzyme (1:500)	66·0	1704

Table 6. *Glucose 6-phosphate dehydrogenase activity of abnormal and normal liver*

The enzyme preparation and incubation mixture is described in the Methods and Materials section; 0·24 μmole of TPN was present.

Time (min.)	TPN reduced (μmoles/g. of frozen tissue): Abnormal liver	Normal liver
0	—	—
2	2·32	0·49
5	3·06	2·78
10	5·36	6·30
15	7·37	6·42
20	8·85	7·73

On the other hand, the diseased tissue was found to possess a high level of phosphoglucomutase activity. The enzyme activity was measured by the method of Najjar (1948), in which an enzyme preparation is incubated with dipotassium α-glucose 1-phosphate and the residual glucose 1-phosphate is estimated by acidic hydrolysis and determination of the liberated inorganic phosphate.

Table 4 shows the rapid action on glucose 1-phosphate in 10 min. at 30°. A further experiment (Table 5), in which the enzyme preparation was diluted (1:10), showed that the substrate is used at a rate of 5088 μmoles/hr./g. of frozen tissue. At higher dilutions (1:100; 1:500), the rate fell to 1536 and 1704 μmoles/hr./g. of frozen tissue respectively [Weber & Cantero (1957) give values for 37°].

Because of the turbidity of homogenates of the diseased liver, a modification of the method of Glock & McLean (1953, 1956) was used for determination of the glucose 6-phosphate dehydrogenase activity of the tissue. In this modification, the enzymic reaction was terminated by the addition of ethanol, and addition of Na_2SO_4 facilitated flocculation of the opalescent substance. The rate of reduction of TPN by the diseased liver was 3·06 μmoles/g. of frozen tissue in 5 min., in contrast with a value of 2·78 μmoles/g. of frozen normal tissue.

A qualitative investigation of the phosphohexoisomerase activities of normal- and diseased-liver homogenates by the method of Glock & McLean (1956) showed that both tissues were highly active.

Glycogen content of liver and other tissues

The content of glycogen in the abnormal muscle and liver was determined by the method of Kemp & Kits van Heijningen (1954) (performed by Dr A. J. M. van Beusekom-Kits van Heijningen). No fixed glycogen was to be found in the muscle, and, in the liver, the amount of glycogen extracted by cold trichloroacetic acid varied between 9·91 and 10·50%, and a further 0·53–0·67% of non-extractable polysaccharide remained. The amount of free sugars (including glucose 1-phosphate) was 0·06–0·09% (Table 7).

The nitrogen content (Kjeldahl) of homogenates of the diseased liver was 21–23 mg. of nitrogen/g. of frozen tissue, whereas for the normal liver the values were 28·6–29·2 mg. of nitrogen/g. of tissue.

DISCUSSION

The present case of glycogen-storage disease is characterized by the accumulation of liver and kidney glycogen which appears to have a 'normal' structure. Glycogen is not deposited in the skeletal muscles, i.e. glycogenesis is not generalized, as in the type 2 disease. Although samples of human liver and kidney glycogen from comparable normal tissues were not available for study, the branching properties of the A.K. glycogens are similar to those of the majority of mammalian glycogens hitherto examined (for a review of glycogen structure, see Manners, 1957).

The interaction of the A.K. glycogens with iodine and concanavalin-A, the average chain length determined by periodate oxidation, the α-amylolysis limit and the low limiting viscosity number differentiate them from amylopectins, and hence eliminate the possibility of a deficiency in amylo-1:4 → 1:6-transglucosidase (branching

Table 7. *Glycogen content of diseased liver (A.K.)*

The pairs of figures represent the results of duplicate experiments.

	Free glycogen* (%)	Fixed glycogen (%)	Total (%)	Free sugars† (%)	
				Anthrone	H_2SO_4 reaction
Immediately deproteinized	10·21, 10·50	0·53, 0·63	10·74, 11·03	0·09, 0·06	0·06, 0·06
Stored for 9 hr. at room temp.	9·91, 10·38	0·60, 0·67	10·58, 10·95	0·09, 0·09	0·09, 0·09

* Values for cold trichloroacetic acid extract corrected for free sugar values.
† 80% (v/v) methanol extracts.

enzyme) as the biochemical lesion (type 4 disease). The extent of β-amylolysis shows that the outer chains contain, on the average, about nine glucose residues and are clearly much longer than those in phosphorylase-limit dextrins. The above reactions are consistent with the view that the present case corresponds to type 1 disease, i.e. a true von Gierke's disease. It follows that the probable enzymic deficiency is of glucose 6-phosphatase and experimental investigations have shown that this is in fact the case.

The previous conclusions (Manners, 1954) on the unusually high degree of branching in the S.K. glycogen have been confirmed. The significantly greater reaction with concanavalin-A, and lower P_M value on α-amylolysis are to be expected; the extremely weak iodine-binding power from measurements of absorption spectra is also of interest. The S.K. and A.K. liver glycogens thus show very marked differences in molecular structure (see Table 2). It is unlikely that these glycogens were markedly changed during their isolation, particular precautions (exclusion of oxygen, etc.) being taken to minimize degradation.

The 'normal' structure of the A.K. glycogen suggests that the biochemical lesion does not reside in a deficiency of amylo-1:6-glucosidase, as has been found by Illingworth & Cori (1952) and Illingworth, Cori & Cori (1956) for some forms of glycogen-storage disease characterized by glycogen accumulation in the heart and kidneys as well as in the liver.

A survey of the activities of glucose 6-phosphatase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase in the diseased liver revealed that the first of these was present in much smaller amounts than in normal human liver from a child of similar age. The other enzymes were highly active. An enzyme preparation from the diseased liver (A.K.) resulted in the liberation of 8·3 μg. of inorganic phosphate/hr./100 mg. of liver from glucose 6-phosphate, compared with 406 μg. with normal liver. Despite unavoidable differences in the age of the diseased and normal tissue, these figures clearly indicate a marked difference in the levels of glucose 6-phosphatase activity. In two cases of the disease reported by Cori & Schulman (1954), values were 20 and 17 μg. of inorganic phosphate liberated/hr. as compared with 362 μg. for normal liver.

The present case thus corresponds to type 1 of Cori's classification, i.e. a case of von Gierke's disease in which the biochemical lesion is a much reduced level of glucose 6-phosphatase. In these circumstances glucose 6-phosphate may be expected to be retained in the liver, not being available to other tissues without removal of the phosphate group. Its conversion into glucose 1-phosphate and hence into glycogen is thus favoured either by the action of the usual phosphorylase mechanisms or by the alternative route suggested by the results of Leloir & Cardini (1957).

The high concentration of free glycogen (10·21; 10·50%) in the frozen diseased liver is characteristic of the disease. The slight decrease in the values (9·91 and 10·38%) after the liver has been kept at room temperature for 9 hr. suggest that little glycogenolysis has occurred during storage of the liver. It has to be said, however, that the diseased liver was not obtained until 50 hr. after the death of the patient and, although the body was refrigerated, the possibility of some changes in glycogen content during this period cannot be excluded. Furthermore, the diseased liver was stored at $-18°$ for 3 months before it was examined. It has not been possible to obtain 'normal tissue' of comparable age and origin.

It is of interest that the present case (A.K.; boy, 4 years old) of type 1 glycogen-storage disease is a sibling (brother) of the patient (S.K.; girl, 12 years old) who suffered from the type 3 disease (Manners, 1954).

Two other cases of siblings having glycogen-storage disease have been reported (Illingworth & Cori, 1952; Cori & Cori, 1952). The first was a girl (M.A.S.) aged 15 months having type 1 von Gierke's disease; a sibling died with similar symptoms though biochemical details were not given. The second was a case of male twins (Daniel B. and Dennis B., aged 3½ years). In both, the structures of the liver glycogens were apparently 'normal', and examination of the liver of Dennis B. showed a decreased level of glucose 6-phosphatase activity. These twins may be presumed to have suffered from

the type 1 form of the disease. Another such family has also been reported by di Sant'Agnese, Andersen, Mason & Bauman (1950).

SUMMARY

1. Liver glycogen ($[\alpha]_D + 196°$ in water) isolated in a case of glycogen-storage disease has a normal structure (average chain length 14·5) and resembles rabbit-liver glycogen in respect of α- and β-amylolysis and the action of isoamylase.

2. The diseased liver has high phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase activities.

3. Glucose 6-phosphatase is present in the diseased liver in much reduced quantity, indicating that the disease corresponds to type 1 of Cori's classification, i.e. von Gierke's disease.

4. The present case (A.K.) is a sibling of one (S.K.) investigated earlier and found to be suffering from type 3 of the disease, i.e. a deficiency of amylo-1:6-glucosidase. It is notable that these siblings suffered from different forms of glycogen-storage disease.

5. The kidney glycogen (A.K.) was also examined and was found to have a normal structure.

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524. *Studies on Carbohydrate-metabolising Enzymes. Part IV.* The Action of Z-Enzyme on Starch-type Polysaccharides.*

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The Z-enzyme contaminant in a barley β-amylase preparation has no action on the anomalous linkages in amylose, but catalyses random hydrolysis of a small number of α-1,4-glucosidic linkages. Amylopectin and amylopectin β-dextrin are also slowly attacked, but under similar conditions, the rate of hydrolysis of glycogen and glycogen β-dextrin is not measurable. The activity, which is optimum at pH 5·6, is stabilised by calcium ions, and partly inhibited by EDTA and mercuric chloride, is attributed to a minute trace of α-amylase.

The Z-enzyme activity of soya-bean β-amylase preparations, and of almond emulsin, is also due to the presence of very small traces of α-amylase.

The methods available for the detection of α-amylase contaminants in carbohydrase preparations are discussed. The most sensitive assay is that using amylose β-limit dextrin as substrate and viscometry as the method of analysis.

THE action of purified β-amylase on most samples of amylose is incomplete, since only 65—80% conversion into maltose is observed.[1] This indicates the presence of a small number of enzymically resistant or anomalous structures in the substrate. Further, amylose is heterogeneous with respect to both degree of polymerisation ($\overline{DP}$) and behaviour on β-amylolysis;[2] with potato amylose of $\overline{DP}$ 3200, 40% (by weight) of $\overline{DP}$ *ca.* 2000 is completely hydrolysed by β-amylase, and the anomalous structures occur only in the remaining material of $\overline{DP}$ *ca.* 6000. For complete amylolysis a second enzyme, named Z-enzyme,[3] is required; this occurs together with β-amylase in soya-beans [3,4] and barley,[5] is inactivated at pH 3·6,[4] and has no action on α-1,3- or α-1,6-glucosidic linkages or on β-glucosidic linkages.[2] We now report evidence that the action of Z-enzyme involves hydrolysis of a small number of non-terminal α-1,4-glucosidic linkages in amylose rather than selective hydrolysis of anomalous linkages and is due to the presence of a trace of an α-amylase in the β-amylase preparation. Z-Enzyme (α-amylase) also slowly degrades amylopectin and its β-dextrin.

In our earlier studies on α-1,4-glucosans,[2,6-8] a highly active preparation of barley β-amylase (Wallerstein Analytical reagent) was used. By conventional tests, α-amylase could not be detected: (*a*) the iodine-staining power of amylopectin β-dextrin measured at 680 mμ did not decrease within 24 hr.;[7] (*b*) the β-amylolysis limit of glycogen was independent of enzyme concentration;[7] (*c*) the molecular weight (13×10^6) of the β-limit dextrin of fœtal sheep liver glycogen was in good agreement with that calculated from the molecular weight (29×10^6) and β-amylolysis limit (49%) of the original glycogen;[9] (*d*) during enzyme action on amylose, the intermediate 50% conversion dextrin had the same molecular size as the original substrate.[10] The same enzyme preparation was considered to contain Z-enzyme since complete degradation of amylose at pH 4·6 but not at 3·6 was observed.[2]

The first indication that the apparent Z-enzyme activity might be due to traces of α-amylase was obtained during studies of the β-amylolysis of amylopectin, when an apparent relation between enzyme concentration and β-amylolysis limit was found.[11] With digests containing 33, 66, 99, and 128 units [12] of β-amylase per mg. of polysaccharide, the apparent β-amylolysis limits were 64, 65, 68, and 71 after 92 hours' incubation at pH 4·9 and 37°. Such a relation is characteristic of the α-amylases.[13] Since this finding was at variance with the previous results,[2,7,9,10] the enzymic homogeneity of the β-amylase preparation was investigated.

* Part III, Gunja, Manners, and Khin Maung, *Biochem. J.*, 1960, **75**, 441.

Degradation of Amylopectin and β-Dextrin by Barley Z-Enzyme.—The above possibility has been examined in detail with amylopectin β-dextrin as substrate, and (*a*) iodine staining, (*b*) viscosity, and (*c*) reducing-power measurements to follow enzyme action.

Peat, Pirt, and Whelan [4] carried out iodine-staining by measuring the decrease in absorption value (A.V.) of the polysaccharide–iodine complex at 680 mμ, the wavelength used for " blue-value " (B.V.) determinations. In our experiments, the wavelength of maximum absorption ($\lambda_{max.}$) has been used, namely, 530—540 mμ. Under these conditions, and with an increase in the relative enzyme concentration and time of incubation, a marked decrease in iodine-staining power was observed. For example, with 0·1% of substrate and 0·2% of barley preparation (equivalent to *ca.* 250 β-amylase units [12] per mg. of polysaccharide) at pH 4·6, an 87% decrease in A.V. occurred in 70 hr., and the residual polysaccharide–iodine complex then had $\lambda_{max.}$ at 420 mμ. This indicates random hydrolysis of non-terminal α-1,4-glucosidic linkages.[14] Under similar conditions the A.V. of glycogen β-dextrin at 470, 430, 420, and 410 mμ was unchanged.

The decrease in A.V. of amylopectin β-dextrin could be detected with only 0·03% barley preparations; at 480, 520, and 560 mμ, the A.V. fell by 17, 30, and 40% respectively after 70 hr. Further experiments (Table 1) showed that this activity was increased by

TABLE 1. *The effect of the barley β-amylase preparation on the A.V. of amylopectin β-dextrin.**

Barley prep. λ (mμ)	Fall in A.V. (%) Normal	Pre-treated with Ca^{++}	Pre-treated with EDTA		Fall in A.V. (%) Normal	Pre-treated with Ca^{++}	Pre-treated with EDTA
480	67	80	14	Original $\lambda_{max.}$ (mμ)	530	530	530
500	75	85	24	Original A.V.	0·67	0·66	0·68
520	82	89	27	Final $\lambda_{max.}$ (mμ) ...	430	430	510
540	85	91	33	Final A.V.	0·22	0·23	0·49
560	86	92	36				
580	88	92	38				

* Digests contained 0·1% of substrate and 0·2% of enzyme preparation and were incubated at pH 4·6 and 35° for 72 hr.

pre-incubation of the enzyme with 5×10^{-3}M-calcium for 30 min., and was decreased by similar treatment with 5×10^{-2}M-ethylenediaminetetra-acetic acid (EDTA). The function of the calcium appears to be that of an enzyme-stabiliser rather than a specific activator. The presence of this ion decreased the rate of inactivation of the enzyme at pH 4·6 and 37° during incubation for 67 hr. in the absence of substrate, and did not restore the activity of a partly inactivated enzyme preparation. EDTA appears to lower the activity by partial removal of the calcium (cf. ref. 15). The protection from inactivation of other α-amylases, including malt α-amylase, by calcium ions has been noted by several workers.[16]

The activity towards β-dextrin was greater in sodium acetate buffer of pH 5·6 than at pH 4·8 or 6·5 (see Figs. 1 and 2); none was detected at pH 3·6. At pH 5·6, the activities in acetate and B.D.H. Universal buffer were identical, in contrast to the behaviour of *Cladophora rupestris* amylase which is more active in the latter buffer.[17] Addition of 5×10^{-3}M-borate, which inhibits isoamylase [18] and activates *Cladophora* amylase,[17] to the acetate buffer had no effect; phenylacetate (7×10^{-3}M) and phosphate (7×10^{-3}M) likewise did not alter the rate of decrease of A.V. (at 540 mμ).

TABLE 2. *Action of normal concentrations of β-amylase on amylopectin.**

	Time of incubation (hr.) 27	49		Time of incubation (hr.) 27	49
	Barley β-amylase			Soya-bean β-amylase	
β-Amylolysis limit (%) ...	55	54	β-Amylolysis limit (%) ...	54	55
A.V. (680 mμ)	0·039	0·036	A.V. (680 mμ)	0·037	0·033
A.V. (540 mμ)	0·152	0·128	A.V. (540 mμ)	0·147	0·136

* Digests contained *ca.* 40 units of β-amylase per mg. of amylopectin.

In contrast to the above results (with *ca.* 250 units of β-amylase per mg. of substrate), the enzyme preparation caused only a slight decrease in the iodine-staining power of amylopectin when experimental conditions similar to those of Peat, Pirt, and Whelan [4] were used [*ca.* 40 units per mg. of substrate; A.V. (680 mμ)] as shown in Table 2. This result illustrates the importance of varying the enzyme concentration when testing for contaminating enzymes.

A slow and limited degradation of 0·5% amylopectin β-dextrin solution by 0·5% barley preparation at pH 5·6 and 25° was also shown by viscosity measurements. After 5, 40, and 70 min., the specific viscosity (η_{sp}) values were 0·194, 0·186, and 0·179 respectively. At pH 3·6, the viscosity was unchanged.

FIG. 1.

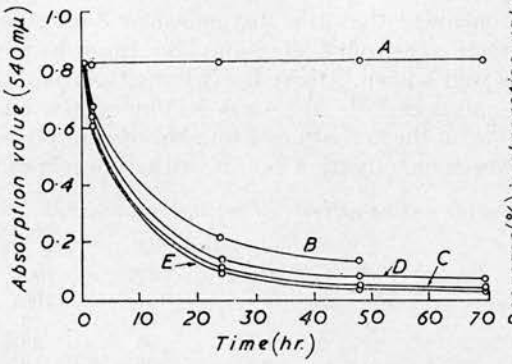


FIG. 2.

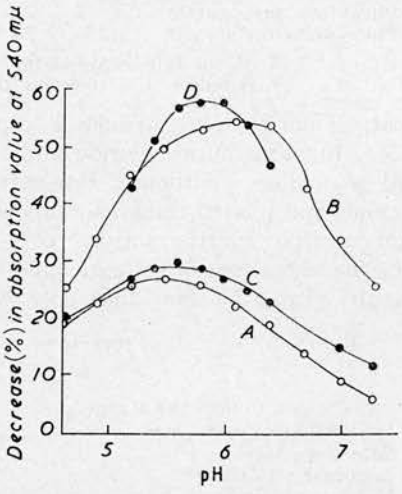


FIG. 1. *Effect of barley Z-enzyme on the iodine-staining power of amylopectin β-dextrin. Enzymic reactions carried out at* pH 3·6 (*A*), 4·6 (*B*), 5·6 (*C*), 6·5 (*D*), *and at* 5·6 *in presence of* 5×10^{-3}M-*borate* (*E*).

FIG. 2. *Effect of* pH *on barley Z-enzyme activity. Substrate: amylopectin β-dextrin;* (*A*, *B*) *acetate buffer analysed after* 6 *and* 24 *hr.;* (*C*, *D*) *phosphate–citrate buffer analysed after* 8·75 *and* 27 *hr.*

Despite the limited degradation of β-dextrin in the above experiments, the effect of this initial α-amylolysis can be magnified by the presence of an excess of β-amylase. Hydrolysis of only a small number of interior α-1,4-linkages liberates non-reducing end-groups which are susceptible to this enzyme. The resultant increase in reducing power is then appreciable, whereas neither β-amylase nor low concentrations of α-amylase, acting separately on β-dextrin, yield measurable amounts of reducing sugars. The results summarised in Table 3 show the apparent percentage conversion into maltose (P_M) during degradation of β-dextrin by the barley preparation. In a further experiment, the relative P_M values at pH 3·6, 5·6, and 6·5 were 4, 45, and 33 respectively.

TABLE 3. *Action of barley preparation on amylopectin β-dextrin.*

	Apparent conversion (%) into maltose *		
Age of digest (hr.)	20	70	86
Digest conditions:			
(*a*) Acetate buffer pH 4·6	25	33	37
(*b*) ,, pH 5·6	35	54	55
(*c*) ,, pH 5·6 (with 5×10^{-3}M-borate)	35	54	56
(*d*) B.D.H. Universal buffer pH 5·6	31	49	52

* For composition of digests, see p. .

Degradation of Amylose and β-Dextrin by Barley Z-Enzyme.—Although the highly branched amylopectin β-dextrin may be used to detect relatively high concentrations of Z-enzyme, the use of a linear substrate is preferable since the hydrolysis of only a small number of linkages will produce a more marked change in physical properties. The effect of certain inhibitors on barley Z-enzyme was therefore investigated by using amylose β-limit dextrin (prepared by the prolonged action of β-amylase at pH 3·6 on potato amylose): the results are summarised in Table 4.

TABLE 4. *Effect of inhibitors * on the action of barley Z-enzyme on amylose β-dextrin.*

Inhibitor	Inhibn. (%) †	Inhibitor	Inhibn. (%) †
10^{-2}M-Iodoacetate	100	10^{-5}M-*p*-Chloromercuribenzoate ...	7
$1{\cdot}5 \times 10^{-5}$M-Mercuric chloride	80	10^{-4}M-Silver nitrate	86
10^{-4}M-Phenylmercuric acetate	79		
10^{-5}M-Phenylmercuric acetate	73		

* All inhibitors caused 100% inhibition of β-amylase.
† Based on A.V. (600 mμ) measurements; see p. .

Peat, Thomas, and Whelan [3] reported that Z-enzyme was not inhibited by *ca.* $1{\cdot}5 \times 10^{-6}$M-mercuric chloride and concluded that this distinguished Z-enzyme from α- and β-amylase. Although the extreme sensitivity of β-amylase towards mercury compounds and related thiol-reactants is well known,[19] there is evidence that thiol groups are not essential for the activity of α-amylases.[20,21] We have examined the effect of various concentrations of mercuric chloride on the activity of a number of α-amylases, and the results (Table 5) show that only *partial* inactivation occurs with concentrations of

TABLE 5. *Effect of mercuric chloride on the activity of α- and β-amylases.*

	Inhibition (%)			
Concn. (M) of $HgCl_2$	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Barley β-amylase *	100	100	100	100
Salivary α-amylase †	84	66	44	22
Bacterial α-amylase †	87	67	35	26
Malt α-amylase ‡	—	96	50	19
Barley Z-enzyme ‡	—	86	70	6

* Assay under Hobson, Whelan, and Peat's conditions.[12]
† Assay under Bernfeld's conditions (*Methods in Enzymology*, 1955, **1**, 149).
‡ See p. .

10^{-5} to 10^{-6}M. Further, the action of barley Z-enzyme is only partially inhibited by mercuric chloride (Table 4 and 5). Notwithstanding the qualitative nature of these studies (the concentration of α-amylase was not identical in the various assays), it is concluded that the use of mercuric chloride shows, in fact, a similarity between Z-enzyme and α-amylase.

p-Chloromercuribenzoate (1×10^{-5}M) completely inhibits β-amylase [19] and yet has less effect on barley Z-enzyme than have similar concentrations of mercuric chloride (Table 4). This reagent has therefore been used for the selective inactivation of β-amylase. On incubation of amylose (0·2%) with barley preparation (0·07%, corresponding to *ca.* 45 β-amylase units/mg.) and *p*-chloromercuribenzoate, a marked decrease in specific viscosity was observed, but without a concomitant decrease in iodine-staining power or increase in reducing power. This is attributed to the random hydrolysis of a small number of non-terminal linkages. Since the iodine-staining power of amylose as obtained by "blue-value" or $\lambda_{max.}$ measurements is approximately the same for samples of $\overline{DP}$ 500 or 2000,[22] this result emphasises the caution required in following *limited* α-amylolysis by iodine-staining. Measurements of the change in reducing power [13] or of the sedimentation constant [10] of the residual amylose are also unsatisfactory when the concentration of α-amylase is extremely low. It is clear that viscometry provides the only sensitive method when concentrations of β-amylase contaminated by Z-enzyme similar to those used in our previous studies [6-8] and by Peat and his co-workers [3,4] are employed.

The presence of traces of α-amylase in unpurified barley β-amylase preparations has been noted by earlier workers including Hopkins, Murray, and Lockwood.[23] The amount of α-amylase appears to depend upon the condition of the grain, and the variety of the barley.[24] Part of the α-amylase may represent precursors of the enzyme which develops in quantity during germination, and part may arise from contamination of the barley husk by amylase-secreting bacteria.[25] The α-amylase constituents of ungerminated and germinated barley are undoubtedly closely related, and we have found that the *initial* action of malt α-amylase on amylose β-dextrin is also not activated by calcium ions, and is partially inhibited by mercuric chloride ($1{\cdot}5 \times 10^{-5}$ and $1{\cdot}5 \times 10^{-6}$M). Further, the optimum pH of unpurified malt α-amylase is *ca.* 5·4,[21] a value similar to that shown in Fig. 2 and different from that of bacterial α-amylase [26] (*ca.* 6·5).

Z-Enzyme Activity of Soya-bean β-Amylase and Almond Emulsin.—Since Z-enzyme was originally detected in unpurified (" stock ") preparations of soya-bean β-amylase [3,4] and in almond emulsin [3,27] (a complex mixture of carbohydrases including β-glucosidases), samples of these have been examined for contamination with α-amylases.

Neither preparation had a significant effect on the production of maltose from amylopectin–β-amylase or Floridean starch–β-amylase systems (cf. Table 2 and ref. 6); by this criterion [13] gross contamination with α-amylase could be ruled out. However, when amylose, amylose β-dextrin, or amylopectin β-dextrin was used as substrate, and assay was by iodine-staining and reducing power or viscosity, the presence of a trace of α-amylase was established. Slight random degradation of the substrates occurred; for example, incubation of amylose (0·1%) with emulsin (0·5%) at pH 4·6 for 24 hr. reduced η_{sp} by 88% and increased the β-amylolysis limit from 75 to 95%. This α-amylolytic activity was increased by calcium ions (which again act as a stabiliser rather than activator), and partly inhibited by EDTA and mercuric chloride (10^{-4}—10^{-6}M), and was optimum in the region pH 5·8—6·1 (cf. Peat, Thomas, and Whelan [3] who reported the action of soya-bean Z-enzyme on amylose β-dextrin as maximum at pH 6). Typical results are shown in Table 6 and Figs. 3 and 4. In similar conditions, the extent of hydrolysis of glycogen

TABLE 6. *Effect of emulsin on the iodine-staining power of amylose β-dextrin.*

	Fall in A.V. (%)			
Wavelength (mμ)	580	600	640	680
Digest conditions : *				
(*a*) Control	56	60	65	69
(*b*) Mercuric chloride: $1{\cdot}5 \times 10^{-5}$M	33	35	38	41
$1{\cdot}5 \times 10^{-6}$M	44	47	55	51
(*c*) Pre-treated with $CaSO_4$ †	72	74	79	82
(*d*) Pre-treated with EDTA: (i) alone †	23	24	26	26
(ii) diluted with water	27	28	31	32
(iii) diluted with $CaSO_4$ †	47	50	51	55

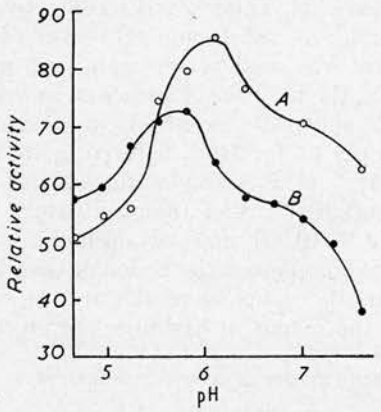
* Incubated for 25 hr. at 35°. † Final concentration 2×10^{-4}M.

β-dextrin by emulsin was approximately one-half that of amylopectin β-dextrin (P_M 3·7 and 7·8 respectively). The properties of the α-amylase present in soya-beans and almond emulsin are therefore generally similar to, although not necessarily identical with, those of barley Z-enzyme.

Discussion and Conclusions.—The recognition of the presence of a trace of α-amylase in the barley and soya-bean β-amylase preparations, and in emulsin, provides an explanation for the observed increase in the β-amylolysis of amylose. The slight random hydrolysis will expose sufficient new non-reducing end-groups to enable further β-amylolysis to take place. If the presence of only one anomalous structure per amylose molecule is assumed, the random hydrolysis of only one α-1,4-glucosidic linkage will cause a 10—15% increase in β-amylolysis limit, *e.g.*, from *ca.* 75 to *ca.* 87%. The ability of Z-enzyme to increase the phosphorolysis limit of amylose, from 70 to 95% conversion into glucose 1-phosphate,[3] can now also be explained in terms of slight α-amylolytic activity.

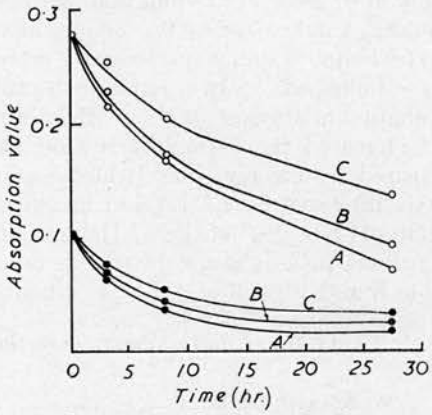
The nature of the structural anomalies in amylose is not yet known.* These may include one or more of the following possibilities: (*a*) an anomalous linkage (*i.e.*, a glucosidic linkage other than the α-1,4-type) in the amylose chain or as a branch point; (*b*) an anomalous residue, *i.e.*, an α-1,4-linked hexose residue derived from D-glucopyranose by substitution with a phosphate group, probably at position 6, or by acylation or oxidation at position 2, 3, or 6; (*c*) both a residue and its linkage may be anomalous. Recent evidence [28] suggests that a small number of glucose residues in amylose may become modified by oxidation during isolation of the polysaccharide, becoming resistant to β-amylase and phosphorylase. Since Z-enzyme is an α-amylase, its action will involve the " by-passing " of such structural anomalies rather than their removal by selective hydrolysis. Similarly, any anomalous linkage which is present as a branch point will not be hydrolysed by Z-enzyme (or any other α-amylase), *i.e.*, Z-enzyme does not act as a " debranching " enzyme.

FIG. 3. *Effect of* pH *on the Z-enzyme activity of emulsin and " stock " soya-bean β-amylase preparation.*



Curve A represents action of soya-bean preparation on amylopectin β-dextrin (reducing-power measurements expressed as P_M values); curve B shows the effect of emulsin on the A.V. (640 *mμ*) *of amylose β-dextrin expressed as percentage decrease.*

FIG. 4. *Effect of soya-bean β-amylase on the* A.V. (540 *mμ*, ○; 680 *mμ*, ●) *of amylopectin β-dextrin.*



Concn. of mercuric chloride in the digests was 0 (*A*), $1{\cdot}5 \times 10^{-6}$M (*B*), *and* $1{\cdot}5 \times 10^{-5}$M (*C*).

Some anomalous structures are present in unfractionated starch since the β-amylolysis limits with purified and " stock " soya-bean β-amylase are 53 and 61% respectively.[4] This difference was attributed to the action of Z-enzyme on the amylose component. However, Hopkins and his co-workers [23] had previously shown that barley β-amylase prepared by a method involving pretreatment at pH 3·4 caused 56% conversion of soluble starch into maltose, and that if this treatment was omitted, or if a trace of bacterial α-amylase was added to the purified preparation, the β-amylolysis limit was 63%. These findings are in accord with our observations, and the view that " Z-enzyme " is a trace of α-amylase adequately explains the effect of pH on the β-amylolysis limit of unfractionated starch (cf. ref. 13).

The amount of α-amylase present in the barley preparation is too small to be assessed accurately but, in comparative experiments, a salivary α-amylase solution containing 34 units [26] was diluted 50,000 times and found to cause a decrease in the iodine-staining power of both amylose and amylopectin β-dextrin and to have only a limited action on glycogen β-dextrin. This suggests that the α-amylase activity of the barley preparation

* Since the proportion of these is extremely low (probably less than 0·1%), their presence in enzymic hydrolysates of amylose cannot be revealed by available methods of analysis, and is inferred from the known specificity requirements of α- and β-amylase.

is of the order of 10^{-3} unit/mg. A further indication of the minute degree of contamination is shown by a comparison of the turn-over number [29] of the related malt α-amylase, equivalent to the hydrolysis of 19,000 bonds per min. per mole, with the limited degradation of amylose β-dextrin observed by viscometry in our experiments during incubation for some hours, and the previous failure to detect degradation of glycogen β-dextrin by light-scattering,[9] or of amylose by sedimentation measurements.[10] It is suggested that the inability of barley Z-enyme to cause appreciable degradation of glycogen is a consequence of (*a*) the low concentration of enzyme and (*b*) the lowered affinity of α-amylases in general for glycogen,[30] rather than to an absolute specificity requirement (cf. R-enzyme which hydrolyses 1,6-linkages in amylopectin but not in normal 12-unit glycogen [14]). The hydrolysis of glycogen or its β-dextrin with normal concentrations of other α-amylases, including preparations from ungerminated barley and soya-beans, can readily be detected.[31]

Our general conclusions are in accord with the findings of Hopkins and Bird,[32] who have emphasised the difficulty in detecting traces of α-amylase when using amylopectin rather than amylose as a substrate, and with the recent results of Baba and Kojima,[33] and of Banks, Greenwood, and Jones.[34] Baba and Kojima also showed the presence of α-amylase in emulsin, and Banks *et al.* independently proved random hydrolysis of starch components, using light-scattering and viscosity measurements, by the Z-enzyme contaminant of several unpurified β-amylase preparations.

It must be noted that the present results do not alter our earlier conclusions [2,6-8] on the molecular structure of starch and glycogen-type polysaccharides, or on the mechanism of β-amylase action,[10] which are derived, in part, from results obtained with the Wallerstein barley β-amylase preparation.

Experimental

Analytical Methods.—The general methods used were those described in earlier papers.[2,6-8] For viscometry, digests were prepared in modified Ubbelohde or Ostwald viscometers, and the viscosity at 25° was measured at intervals. Since the activity of an α-amylase is related to $d(1/\eta_{sp})/dt$,[35] graphs of $1/\eta_{sp}$ against t were prepared. With identical enzyme and substrate concentrations, the effect of added reagents could then be observed by a comparison of the slopes. The polysaccharide solutions were filtered through sintered glass (G4) before analysis.

In the iodine-staining experiments with amylopectin β-dextrin, measurements at 540 mμ increased the A.V. to *ca.* 0·5 in the most sensitive region of the spectrophotometer (Unicam S.P. 500) (cf. A.V. of *ca.* 0·1 at 680 mμ).

Enzyme Preparations.—The properties of the barley β-amylase are reported in ref. 7. " Stock " and purified soya-bean β-amylase were prepared by the methods of Bourne, Macey, and Peat [36] and Peat, Pirt, and Whelan.[4] Sweet-almond emulsin was isolated by Tauber's method; [37] a weighed amount was centrifuged in the stated volume of water, and insoluble material was discarded. Wallerstein malt diastase was used as a source of malt α-amylase, with short incubation periods to minimise the effect of β-amylase.

Substrates.—(a) *Amylose.* Various samples made by the fractionation of starch from potatoes (var. Kerr's pink) with thymol and butanol were used, together with amylose VI, VII, and VIII.[2] Amylose β-dextrin was prepared by incubating amylose VIII (500 mg.) with barley β-amylase (100 units/mg.) at pH 3·6 for 24 hr. The β-amylolysis limit was 72%. The digest was heated for 10 min., cooled, and filtered (G4 sinter), and the pH was adjusted to 5·6 with aqueous sodium hydroxide. The dextrin was stored under toluene at room temperature; the maltose present did not interfere with subsequent measurements.

(b) *Amylopectin.* Fractionation of potato starch with thymol or pyridine gave samples I and II respectively. Waxy-maize and sorghum starch were commercial samples. Amylopectin β-dextrin was prepared from waxy-maize starch I (5 g.) treated with purified β-amylase (6000 units) in a total volume of 250 ml. at 35° for 48 hr. The β-amylolysis limit was 53%. After dialysis, the dextrin was isolated by freeze-drying. Samples of amylopectin β-dextrin were also prepared from waxy-sorghum starch and potato amylopectin by similar methods.

(c) *Glycogen β-dextrin.* This was isolated from a digest of *Ascaris lumbricoides* glycogen and β-amylase.

Action of Barley Z-Enzyme on Amylopectin β-dextrin.—(a) *Iodine-staining measurements.* Polysaccharide (25 mg.), barley preparation (52 mg.), 0·2M-sodium acetate buffer (pH 4·6; 3 ml.), and water to 20 ml. were incubated at 35° for 70 hr. Samples (2 ml.) were removed, heated to inactivate the enzyme, and stained with iodine solution (0·2% in 2% potassium iodide solution; 2·5 ml.) in a total volume of 25 ml. The A.V. (540 mμ) of amylopectin β-dextrin decreased from 0·740 to 0·097 and the product showed $\lambda_{max.}$ 420 mμ and $A.V._{max.}$ 0·210. With glycogen β-dextrin, the initial and final A.V.'s were: at 470 mμ, 0·044 and 0·042; at 430 mμ, 0·073 and 0·074; at 420 mμ, 0·075 and 0·078; at 410 mμ, 0·067 and 0·069. When only 6·25 mg. of barley preparation were used, the following results were obtained:

λ (mμ)	480	500	520	540	560	580
Initial A.V.	0·545	0·622	0·674	0·680	0·630	0·552
Final A.V.	0·446	0·469	0·472	0·440	0·377	0·319

Barley preparation (*ca.* 50 mg.), pretreated with 5×10^{-3}M-calcium sulphate or 5×10^{-2}M-EDTA (pH 4·7) for 30 min. at 37°, was incorporated into similar digests. Samples (3 ml.) were removed after 72 hr.; the results are reported in Table 1. In a further experiment with 0·67 mg. of EDTA-treated enzyme, only a slight decrease in iodine-staining power was noted:

λ (mμ)	480	500	520	540	560	580
Initial A.V.	0·529	0·607	0·675	0·665	0·610	0·531
Final A.V.	0·529	0·573	0·600	0·575	0·517	0·436

The optimum pH for EDTA–calcium complex formation is 7·5;[38] hence, in the above experiments, the calcium ions may not have been completely removed.

(b) *Measurement of reducing power.* Digests (20 ml.) were prepared containing amylopectin β-dextrin (19·2 mg.), barley β-amylase (3800 units), buffer solution (3 ml.), and water. At intervals samples (5 ml.) were deproteinised, and the apparent maltose contents were determined. The results are shown in Table 3.

(c) *Viscometry.* Amylopectin β-dextrin (1% filtered solution; 10 ml.), 0·2M-acetate buffer (5 ml.) of pH 3·6 or 5·6, and barley β-amylase (100 mg. in 5 ml. of water) were mixed in a viscometer. η_{sp} was determined during 2 hr. At pH 5·6, $d(1/\eta_{sp})/dt$ indicated[35] a relative activity of $7{\cdot}8 \times 10^{-3}$ unit, and in presence of mercuric chloride ($1{\cdot}5 \times 10^{-5}$M), of $7{\cdot}2 \times 10^{-3}$ unit. At pH 3·6, there was no change in viscosity.

Effect of pH *and Various Ions on Activity.*—Amylopectin β-dextrin (*ca.* 30 mg.), barley preparation (6000 units), buffer (3 ml.), and water (to 25 ml.) were incubated at 35°. The buffers used were 0·2M-acetate of (*a*) pH 3·6, (*b*) pH 4·8, (*c*) pH 5·6, (*d*) pH 6·5, and (*e*) pH 5·6 containing borate to give a final concentration of 5×10^{-3}M. The A.V. of samples (2 ml.) was measured at intervals. The results obtained at 540 mμ are shown in Fig. 1. Similar results were obtained over the range 460—680 mμ.

For the pH–activity curves, β-dextrin (10 mg.) was incubated with β-amylase (1250 units) and 0·2M-acetate buffer (pH 4·6—7·6; 5 ml.) in a total volume of 15 ml. Samples (3 ml.) were removed after 6 and 24 hr. and the A.V.'s at both 540 and 680 mμ were determined. After 6 hr., at 540 mμ, the maximum decrease was at pH 5·5; after 24 hr., over the range pH 6·1—6·4 (see Fig. 2). The small change in pH is attributed to the decreased stability of the enzyme in acetate buffer at pH 4—6. The same results were obtained from A.V. determinations at 680 mμ. The experiment was repeated with phosphate–citrate buffer (pH 5·2—7·3; 0·1M-citric acid and 0·2M-disodium hydrogen phosphate; 3 ml.) in a 10 ml. digest. The maximum fall in A.V. (540 mμ) occurred at pH 5·6 after 8·75 hr. and at pH 5·8 after 27 hr.

Digests containing β-dextrin (10 mg.), barley preparation (2000 units), and buffer (5 ml.) in a total volume of 15 ml. were incubated at 35°. The following results were obtained.

Buffer	Acetate (pH 5·6)	B.D.H. Universal (pH 5·6)	Phenyl-acetate *	Borate *	Phosphate *
Fall (%) in A.V. (540 mμ):					
after (*a*) 6 hr.	34	33	34	33	33
,, (*b*) 27 hr.	70	69	70	70	70
P_M, after 99 hr.	12·6	12·9	13·1	12·5	12·6

* These digests contained 5 ml. of sodium acetate buffer (pH 5·6) and 5 ml. of 2×10^{-2}M-anion.

The function of the calcium ion was examined by incubating enzyme solution (2 ml.) with β-dextrin (10 mg.) and acetate buffer (pH 5·8; 3 ml.) in a total volume of 10 ml. Digest no. 1

contained barley preparation pre-incubated at 37° and pH 5·8 for 67 hr.; digest no. 2 contained enzyme solution as above, but also 5×10^{-2}M-calcium acetate; digest no. 3 was as digest no. 1 except that the enzyme was added to a mixture of β-dextrin and calcium acetate.

Fall (%) *in* A.V. (540 mμ).

Incubation (hr.)	4·5	7	24
Digest no. 1	13	18	49
Digest no. 2	31	45	83
Digest no. 3	13	19	51

Action of Barley Z-Enzyme on Amylose β-Dextrin.—(a) *Iodine-staining measurements.* Digests containing amylose β-dextrin solution (0·46 mg./ml. by acid hydrolysis; 14 ml.) and β-amylase (14 mg. in 0·5 ml. of water; pre-incubated at 20° for 20 min. with 0·5 ml. of inhibitor solution) were incubated at 35°. Samples (4 ml.) were withdrawn after 2·5 and 4·5 hr., stained with iodine solution (1 ml.), and diluted with water to 25 ml. A.V.'s were measured at 560, 580, 600, and 640 mμ. The trend of results was the same at all wavelengths; the results at 600 mμ are given in Table 4.

The effect of mercuric chloride was examined in digests containing amylose β-dextrin (10 mg.), β-amylase (15 mg.), 0·2M-acetate buffer (pH 5·5, 3 ml.), mercuric chloride solution (1 ml.), and water (6 ml.). Samples (3 ml.), removed after 2·5 hr., gave the following results:

Concn. of $HgCl_2$ (M)	10^{-4}	10^{-5}	10^{-6}	Nil
Decrease (%) in A.V. at (600 mμ)	7	15	46	49

(b) *Viscometry.* A digest containing amylose solution (70 mg.; 20 ml.; with *p*-chloromercuribenzoate, 10^{-5}M) and β-amylase (25 mg. in 15 ml. of 0·2M-acetate buffer of pH 4·6; with *p*-chloromercuribenzoate, 10^{-5}M) was prepared in a viscometer. The following results were obtained:

Time (min.)	15	30	60	97	120	155	205	20 hr.	45 hr.
$1/\eta_{sp}$	3·13	3·32	3·73	3·94	4·20	4·48	4·65	6·25	6·76

Samples were also removed for the measurement of A.V. at both 540 and 680 mμ, and of the reducing power. No change was detected within 24 hr. A control experiment showed that 10^{-5}M-*p*-chloromercuribenzoate had no effect on the reaction of maltose with the Somogyi reagent; in the absence of this material the viscosity change is accompanied by a marked decrease in iodine-staining power and rapid production of reducing sugars.

Action of Normal Concentrations of β-Amylase on Amylopectin.—Digests were prepared containing waxy-maize starch I (30 mg.), 0·2M-acetate buffer (pH 4·6; 10 ml.), barley β-amylase or " stock " soya-bean β-amylase (1300 units), and water to a final volume of 50 ml. Samples (2 ml. for iodine-staining; 3 ml. for reducing-power measurements) were removed at intervals. The results are in Table 2.

Action of " Stock " Soya-bean β-Amylase on Amylopectin β-Dextrin.—Polysaccharide (23·9 mg.), 0·2M-acetate buffer (pH 4·6; 3 ml.), enzyme solution (3 ml.), and water (19 ml.) were incubated at 35°. [The enzyme solution was prepared by dissolving 50 mg. of powder (activity *ca.* 100 units/mg.) in 5 ml. of buffer and centrifuging the mixture.] Samples (2 ml.) were removed after 27 and 72 hr.: the results after 27 hr. were:

λ (mμ)	480	500	520	540	560	580	680
Initial A.V.	0·528	0·612	0·687	0·700	0·649	0·574	0·198
Final A.V.	0·034	0·039	0·039	0·038	0·038	0·038	0·020

Similar results were obtained after 72 hr.

The effect of pH was examined in digests containing β-dextrin (5 mg.), phosphate–citrate buffer (pH 4·6—7·6; 5 ml.), 1% β-amylase solution (2 ml.) and water (3 ml.). The P_M values of 3 ml. portions were determined after 25 hr. at 35° (see Fig. 3).

Amylopectin β-dextrin (10 mg. in 5 ml. of water) was added to 0·3% β-amylase solution (4 ml.) pre-incubated as follows: (*a*) with 0·2M-acetate buffer (pH 6·1; 10 ml. containing 5×10^{-3}M-calcium acetate); (*b*) with buffer containing 5×10^{-3}M-EDTA; (*c*) with buffer alone. The total volume was 24 ml. The decreases (%) in A.V. (540 mμ) of samples (2 ml.) measured after 1, 13, and 42 hr. were: (*a*) 7, 66, and 92; (*b*) 0, 6, and 13; (*c*) 1, 24, and 50 respectively. The P_M values after 42 hr. were 19, 7, and 14 respectively.

Digests containing β-dextrin (10 mg.), 0·2M-acetate buffer (pH 6·0; 5 ml.), 0·25% β-amylase

solution (2 ml.), water, and mercuric chloride (to give final concentrations of $1{\cdot}5 \times 10^{-5}$ and $1{\cdot}5 \times 10^{-6}$M severally) in a total volume of 15 ml. were prepared. The change in A.V. (540 and 680 mμ) is shown in Fig. 4. The P_M values determined after 8·5 and 27·5 hr. were: (*a*) without mercuric chloride, 15 and 18; (*b*) $1{\cdot}5 \times 10^{-6}$M, 14 and 18; (*c*) $1{\cdot}5 \times 10^{-5}$M, 5 and 9.

Action of " Stock " Soya-bean β-Amylase on Other Polysaccharides.—Potato amylopectin II (40 mg.), 0·2M-acetate buffer (pH 4·6; 9 ml.), 0·2% β-amylase solution (1 ml.), and water to 30 ml. were incubated at 35°. The β-amylolysis limit was 49 (0·5 hr.), 50 (1 hr.), 53 (4 hr.), and 53 (24 hr.). In similar conditions soluble starch had a β-amylolysis limit of 62% but with purified soya-bean β-amylase the value was 57%. When potato amylose VI was used, β-amylolysis limits of 77% were found after 4 and 22 hours' incubation with *ca.* 5 units per mg. of polysaccharide at pH 4·6; with higher enzyme concentrations, complete degradation occurred.

Action of Emulsin on Amylopectin.—Waxy-maize starch (20 mg.), barley β-amylase (50 units/mg.), 0·2M-acetate buffer (pH 5·0; 4 ml.), and water (to 25 ml.) were incubated at 35° for 48 hr. The β-amylolysis limit was 57%. Emulsin (20 mg.) was added; after a further 24 hr. the β-amylolysis limit was 58%. In a second digest in which β-amylase and emulsin acted together on waxy-maize starch, the β-amylolysis limit was 56 and 56% after 24 and 48 hr.

Action of Emulsin on Amylose.—Amylose VIII (30 mg.) was incubated at pH 3·6 with barley β-amylase (100 units/mg.) in a total volume of 50 ml. for 24 hr. The β-amylolysis limit was 75%. The enzyme concentration was then doubled, and after 24 hr. the β-amylolysis limit was 76%. The pH of the digest was then adjusted to 4·8, and to a 15 ml. portion 1% emulsin solution (5 ml.) was added. After 1 and 24 hr., the β-amylolysis limits were 88 and 95% and the A.V.'s (680 mμ) (measured on a 3 ml. sample stained with 1 ml. of iodine solution and diluted to 25 ml.) were 0·005 and 0·002 respectively compared with an original A.V. of 0·200.

A second 15 ml. portion of the digest was incubated with emulsin and 0·01M-mercuric chloride (0·5 ml.) in a total volume of 25 ml. (final concentration 2×10^{-4}M). The A.V.'s (680 mμ) were 0·202, 0·195, and 0·165 after 0, 1, and 24 hr. respectively.

For amylose VI, β-amylolysis limits of 73% before, and 101% after, addition of emulsin were obtained; the A.V. (680 mμ) of a sample fell from 0·21 to 0·08.

Amylose VII solution (2 mg./ml.; 25 ml.) was then incubated at pH 4·6 with 2% emulsin solution (12·5 ml.) in a total volume of 50 ml. After 24 hr. the digest was heated, then cooled, and denatured protein was removed at the centrifuge. The residual polysaccharide was precipitated with ethanol, washed, and dried. The specific viscosity at 25° of 25 mg. of polysaccharide dissolved in 20 ml. of 0·2N-potassium hydroxide was 0·025, and the β-amylolysis limit at pH 3·6 was 93%. Under similar conditions, amylose VII has a β-amylolysis limit of 75%.[2] In a control experiment with heat-denatured emulsin, the residual polysaccharide had a specific viscosity of 0·212.

Effect of pH *on Activity.*—Digests containing amylose β-dextrin (2·4 mg. in 2 ml. of water), phosphate–citrate buffer (pH 4·6—7·6; 2 ml.), and emulsin solution (15 mg. in 1 ml. of water) were incubated at 37°. Control digests (*a*) without enzyme and (*b*) without β-dextrin were also prepared. After 27 hr. samples (2·5 ml.) were withdrawn, heated, and centrifuged. Iodine solution (1 ml.) was added to 2 ml. of solution and the A.V.'s at 640 mμ were measured after dilution to 25 ml. The results are shown in Fig. 3. No correction was required for the enzyme control.

Effect of Calcium Ions and Inhibitors on Emulsin.—(a) *Iodine-staining measurements.* Digests were prepared containing 0·4% amylose solution (5 ml.), emulsin (0·5% in 0·2M-acetate buffer of pH 5·6; 5 ml.), and calcium acetate (5×10^{-3}M; 0·5 ml.) or water (0·5 ml.). Digest (*a*) contained newly prepared enzyme and substrate and water; digest (*b*) contained enzyme pre-incubated at 37° for 40 hr.; digest (*c*) contained enzyme pre-incubated with calcium, and digest (*d*) contained pre-incubated enzyme added to calcium. Samples (2 ml.) were withdrawn at intervals, heated, and coagulated, protein was removed, and 1 ml. portions were used for

Fall (%) *in* A.V.

Incubation (hr.)	(*a*)	(*b*)	(*c*)	(*d*)	Incubation (hr.)	(*a*)	(*b*)	(*c*)	(*d*)
5	11	—	—	—	21·5	—	9	29	12
6·5	—	2	4	2	40	52	—	—	—
16	35	—	—	—	47·3	—	12	47	15

A.V. (680 mμ) measurements. Results, tabulated, show that the calcium ions have a stabilising rather than an activating action.

(b) *Viscosity measurements.* Digests containing amylose β-dextrin (13·5 mg.), ~2% emulsin solution (4·5 ml.; pre-incubated for 20 min. with reagent), and water (total volume 15 ml.) were incubated in a viscometer. The relative activities [35] in two series of experiments were (*a*) 6·5 and 5·7 × 10^{-3} unit with water and mercuric chloride (1·5 × 10^{-6}M) respectively, (*b*) 9·3, 8·2, and 6·5 × 10^{-3} unit with water, calcium sulphate (2 × 10^{-4}M), and EDTA (2 × 10^{-4}M) respectively.

Action of Emulsin on Amylopectin and Glycogen β-Dextrin.—Digests were prepared containing either amylopectin β-dextrin (30 mg. in 15 ml. of 0·2M-acetate buffer of pH 5·8) or glycogen β-dextrin (20 mg. in 5 ml. of buffer) and 0·5% emulsin (5 ml.). The annexed results were obtained.

	Amylopectin β-dextrin		Glycogen β-dextrin
Time of incubation (hr.)	Decrease (%) in A.V. (540 mμ)	P_M	P_M
4	7	—	—
24	26	4·2	—
30	—	—	2·4
48	35	7·8	3·7

Action of Malt α-Amylase on Amylose β-Dextrin.—β-Dextrin (5 mg.), 0·2M-acetate buffer (pH 5·6; 9 ml.), 0·01% diastase solution (0·5 ml.), and water or reagent (0·5 ml.) were incubated at 37° for 30 min. The A.V. (640 mμ) of a sample (3 ml.) was then determined. The following results were obtained (expressed as % fall in A.V.); control, 68; calcium acetate (2·5 × 10^{-4}M), 67; EDTA (2·5 × 10^{-3}M), 66; mercuric chloride (1·5 × 10^{-4}M), 2; (1·5 × 10^{-5}M), 25; (1·5 × 10^{-6}M), 41; *p*-chloromercuribenzoate (10^{-5}M), 51. The last observation shows the effect caused solely by the α-amylase, and the results in Table 5 are calculated on this basis.

Action of Salivary α-Amylase on β-Dextrins.—Freeze-dried salivary α-amylase (34 units; [26] 1 mg. in 1 ml. of water) was diluted 50,000 times, and sodium chloride was added to a final concentration of 0·05M. Digests were prepared containing various β-dextrins (4·8—12·0 mg.) dissolved in 0·2M-acetate buffer of pH 5·8 (6 ml.) and diluted salivary amylase (1 ml.). Samples (1 or 3 ml.) were removed for analysis by iodine-staining or reducing-power measurements. After incubation for 23 and 42·5 hr., the following results were obtained: with amylose β-dextrin (4·8 mg.), the A.V. (640 mμ) fell by 25 and 53%; with amylopectin β-dextrin (12·0 mg.), the A.V. (540 mμ) fell by 24 and 39%; with glycogen β-dextrin (12·0 mg.), the P_M values were 0·9 and 1·8 respectively.

In additional digests containing amylopectin β-dextrin (17·0 mg.) and glycogen β-dextrin (17·8 mg.), and either (*a*) 6·8 × 10^{-4} unit of α-amylase or (*b*) 13·6 × 10^{-4} unit, in a total volume of 16—27 ml., the extents of degradation were:

	Incubation (hr.)	
Amylopectin β-dextrin	24·5	48·5
(*a*) Fall (%) in A.V. (540 mμ)...	10	22
P_M	3·0	3·2
(*b*) Fall (%) in A.V. (540 mμ)...	22	42
P_M	6·2	7·0

	Incubation (hr.)	
Glycogen β-dextrin:		
(*a*) P_M	0·5	1·4
(*b*) P_M	1·4	3·2

Under similar conditions, the diluted salivary α-amylase thus hydrolyses more than twice as many bonds in amylopectin β-dextrin as in glycogen β-dextrin.

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Observations on the Absorption Spectra of Polysaccharide Iodine Complexes. By A. R. ARCHIBALD, D. J. MANNERS and A. WRIGHT. (*Department of Chemistry, University of Edinburgh*)

In the course of structural studies on starch-type polysaccharides, we have measured the absorption spectra of the iodine complexes of more than 50 different samples of amylopectin and glycogen. Using conditions similar to those of Peat, Whelan, Hobson & Thomas (1954), in which the absorption spectra of 0·01 % polysaccharide solution stained with a solution of 0·02 % iodine in 0·2 % potassium iodide is measured in a Unicam SP. 500 spectrophotometer, values for the wavelength of maximum absorption ($\lambda_{max.}$) and the extinction (absorption value) at this wavelength ($E_{max.}$) have been obtained.

Glycogens from various biological sources gave $\lambda_{max.}$ values in the range 420–490 mμ with $E_{max.}$ 0·1–0·4. The iodine-staining power increased generally in the order glycogen β-limit dextrin, invertebrate glycogen, mammalian-liver glycogen and mammalian-muscle glycogen, but there was no correlation between $\lambda_{max.}$ or $E_{max.}$ values and the degree of branching, or the relative lengths of the exterior and interior chains.

With amylopectins, the corresponding $\lambda_{max.}$ and $E_{max.}$ values were 530–550 mμ and 0·8–1·2, respectively, and there was no apparent relation between iodine-staining power and branching characteristics. Floridean starches gave absorption spectra with $\lambda_{max.}$ about 500 mμ and $E_{max.}$ about 0·7.

Amylopectin and glycogen differ markedly in iodine-staining properties in two additional respects. First, during the β-amylolysis of glycogen, a decrease in both $\lambda_{max.}$ and $E_{max.}$ is observed, whereas for amylopectin the $\lambda_{max.}$ of the intermediate and limit dextrins is unaltered. The type of iodine binding in the two polysaccharides must therefore differ, and in the case of amylopectin it is suggested that the length of the interior chains is a controlling factor. Secondly, the addition of ammonium sulphate (cf. Schlamowitz, 1951) to the polysaccharide–iodine–iodide solution caused an increase in iodine-staining power. With amylopectins, the $\lambda_{max.}$ is unaltered but $E_{max.}$ is increased by 10–30 %; with glycogens, $\lambda_{max.}$ is increased by about 40 mμ, and $E_{max.}$ is increased to the range 0·8–1·1. In these solutions, the dehydrating action of ammonium sulphate may facilitate the binding of iodine by a proportion of the exterior chains of glycogen.

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